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CUTANEOUS STREPTOTHRICOSIS OF CATTLE IN NIGERIA

II. The Aerobic Actinomycete (Nocardia sp.) Associated with the Lesions

By

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INTRODUCTION

In domestic animals there are a number of important skin diseases characterised primarily by a proliferative dermatitis and the presence within the lesions of branched, filamentous organisms. The latter produce a Gram-positive mycelium which parasitises keratinising epithelial cells and gives rise to coccal forms, typically arranged in two to eight parallel rows within the parent filaments.

In Australia (Bull, 1929) South Africa (Steyn, 1931) and, more recently, in Great Britain (Kane, Downing and Wilsdon, 1955), infections of the fleece-bearing parts of the skin of sheep are known as "lumpy-wool disease" or mycotic dermatitis (Toop, 1943). In Australia the latter name has also been applied to similar skin infections of cattle and horses (Albiston, 1933; Edgar and Keast, 1940; Moule and Sutherland, 1947). Schultz (1955) and Chodnik (1956), both working in Africa, also used the term "mycotic dermatitis" for a disease of cattle which is more commonly called "cutaneous streptothricosis" (Hudson, 1937; Malfroy, 1938; Curasson and Mornet, 1941; Buck, 1948; Mornet and Thiery, 1955; Zlotnik, 1955). Earlier names for the same condition included contagious dermatosis, dermatitis or impetigo (van Saceghem, 1914, 1915, 1916; Hornby, 1920), dermatomycosis (Reid, 1921) and bovine tropical impetigo (van Saceghem, 1934).

In Great Britain a condition affecting the hairy parts of the skin of wooled sheep is known as "strawberry foot-rot" or proliferative dermatitis. It was, until recently, regarded as of viral origin (Selbie, 1945; Abdussalam and Blakemore, 1948; Harriss, 1948; Horgan and Haseeb, 1948). Thompson (1954) showed that it could be transmitted by pure cultures of an organism isolated from the lesions, which is morphologically identical to those of "cutaneous streptothricosis" or lumpy-wool disease. His observation has been confirmed by Nisbet (1954) and Nisbet and Bannatyne (1955) who called the disease "actinomycotic dermatitis". Lall (1949) used the same name for a disease, which was in many respects similar, affecting sheep in India.

This brief survey indicates the wide distribution of diseases caused by the same or closely related organisms in the skin of domestic animals. Whilst leaving out of account a host of vernacular names, (Beaton, 1955), with nothing to recommend them scientifically, the number of different designations employed reflects the lack of agreement about the classification and properties of the causal agents.

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This paper describes the cultural properties of Nigerian strains of the organism of bovine cutaneous streptothricosis showing a very similar behaviour to organisms isolated from the other conditions mentioned above.

MATERIALS AND METHODS

To obtain smears or material for culture well-developed lesions on the skin of naturally infected cattle were selected (Plowright, 1956). Scabs of 5 to 10 mm. minimum diameter were lifted carefully, either manually or with forceps, and the underlying yellowish, cheesy material was smeared on slides or collected aseptically. Where scabs were collected in screw-cap containers they rapidly dried out and became hardened, but a suitable inoculum for cultures could still be obtained by moistening the concave (under) side with sterile saline and scraping away the softened material with a flamed scalpel. Some samples found positive on smear were negative on culture, as heavy contamination by some organisms, *Staphylococcus albus* and coliforms in particular, completely suppressed the development of Nocardia colonies.

For primary isolation 5 per cent ox-blood agar plates were found to be most suitable; individual colonies could easily be identified at 24 to 48 hours by the use of a stereoscopic microscope. With the unaided eye or hand lens it was difficult or impossible to distinguish clearly either the aerial mycelium or deep ramifications of the colonies.

For the maintenance of strains it was discovered that no single medium allowed the retention of the original growth vigour. Sub-cultures on solid media, nutrient, serum, or blood-agar, required to be alternated with others in liquid media such as meat-infusion, serum or glucose broth. Even with this precaution, cultures sometimes died out rapidly after 7 to 10 days at room temperature or 37°C and after slightly longer periods on slopes in sealed tubes at 4°C. Inoculation on to the skin of rabbits appeared to restore the vigour rapidly in the first passage. Freeze-dried organisms retained their viability in vacuo for at least 9 months. In general, the cultural reactions of the strains investigated were tested before their 10th successive passage in artificial media. The media employed were all prepared according to standard methods (Mackie and McCartney, 1948).

Of the stains used for smears (Gram's, methylene blue, Giemsa, carbol thionin) Gram's gave better results than any other and was used as a routine.

RESULTS

Appearance of the Organism in Lesion Smears

There is nothing to add to the descriptions given for similar material by van Saceghem (1914, 1916), Bull (1929), Steyn (1931), Hudson (1937), Lall (1949) and Thompson (1954). Filaments were not strictly-speaking acid-fast, although segments of the mycelium occasionally showed some resistance to decolorisation by 1 per cent acetic acid. Filaments and cocci were usually plentiful in relatively uncontaminated lesions and a firm diagnosis could be made on the basis of their joint morphology. Where a wet, diffuse, eczematous or gangrenous condition had ensued it was sometimes difficult to detect characteristic filaments among the mass of contaminants.

The appearance and distribution of the organism in fixed tissues will be described later.

Cultural and Biochemical Characteristics

Solid media. After seeding ox-blood-agar plates, at 18 to 24 hours isolated colonies were minute, with a rough, matt surface and radial outgrowths on the surface of the medium. With heavy inocula there were some areas of smooth, white heaped-up growth and elsewhere a dull greyish film; short, unbranched aerial hyphae were invariably present on careful microscopic examination. At 48 hours the centre of isolated colonies was raised, white and glistening but rough feathery projections, with a few aerial hyphae, occurred on the surface of the agar. There was typically a narrow zone of β-haemolysis which reached 1 mm. in radius. After 72 to 96 hours smooth colonies reached 1 mm. in diameter and short spiky outgrowths sometimes persisted. There was marked depression of the colony edge and increasing amounts of a yellowish or orange pigment. Some colonies remained totally rough and translucent, often with almost vertical edges and rounded or irregular shapes; these were not pigmented. At 6 to 10 days the pigmentation was becoming brownish and there were numerous daughter colonies on the surface of the primary growth. These often grew rapidly to dwarf the parent colony. Their shape was irregular, even bizarre, some were rough, translucent and coral-like, others rounded and glistening. No haemolysis was observed on 5 per cent horse or rabbit-blood-agar plates.

On 10 per cent ox or horse serum-agar plates growth was less vigorous and the aerial mycelium poorly developed. After 7 to 10 days at 37°C, there were many profuse downgrowths into the medium extending to about 1 mm. depth and 1 to 1·5 mm. radius and giving a puffball appearance. Other colonies were confined to the surface, with roseate edge, folded surface and orange-yellow or salmon-pink pigmentation. On 1 per cent glucose-agar plates there were only a few colonies on the primary streaks. The majority were rough, coral-like, irregular in shape and with no aerial mycelium, but a distinct yellowish pigmentation. A similar meagre growth was seen on 7 per cent glycerine agar. On agar or serum-agar slopes in universal containers, fine floccules appeared in the water of condensation: surface growth was more rapid than in unsealed vessels and extended on to the glass. A wrinkled, dry, continuous film with depressed margin followed heavy inocula. Mature, isolated colonies had a central, raised, pigmented nodule, with a flat surface outgrowth and profuse downgrowth into the medium. Cultures were catalase-negative. One per cent glucose-agar shakes produced isolated, rounded or irregular colonies, extending down to the bottom of the medium which was 4 to 5 cm. deep. Growth was almost completely suppressed on MacConkey-agar plates.

Gelatin stabs showed a heavy flocculent growth after 48 hours at 37°C. Three of four strains tested produced liquefaction at 13 days, the other failed to do so after 30 days.

On inspissated-serum slopes a dull yellowish film or isolated colonies, up to 1·5 mm. in diameter were seen after 7 days. Ten strains isolated in 1954 produced liquefaction, visible at the 3rd day, which was almost complete at 7 to 10 days. This result was reproducible. There was only slow liquefaction with 5 strains isolated in 1955; in a repeat test only one strain of the five produced any liquefaction after 30 days; no reason for this irregularity was found (Table 1). Dorset-egg slopes showed dry, greyish colonies or confluent growth and floccules in the water of condensation at 7 days. After 22 to 30 days there was yellow or orange pigmentation with no digestion of the medium.

Litmus milk was coagulated after 48 hours, followed by digestion of the clot, peptonisation and some decolorisation at 10 days.

Liquid media. In nutrient broth there was slight opacity with suspended floccules at 24 hours. Later an increasing opacity and deposit of coarse floccules was seen, with sometimes a ring at the liquid surface or a pellicle breaking up into fragments on shaking. Occasionally suspended filaments extended from the surface to the deposit or there was a film-like growth on the glass. Growth in 10 per cent serum broth was often less profuse. Sometimes a few "puffball" colonies only were seen, with no opacity. In 1 per cent glucose-broth and 5 to 7 per cent glycerine-broth, growth occurred as in plain broth or was less profuse. 1 per cent peptone water produced a good growth of loose floccules with slight opacity of the medium. There was no production of indole.

Fermentations of the organism were tested in a peptone-water base. With glucose and fructose there was acid with no gas at 48 hours. Sucrose, galactose, maltose, inulin, lactose, xylose, sorbitol, dulcitol, inositol, mannitol, glycerol and salicin were not fermented after 30 days (Table 2). No growth was obtained in a standard Czapek-Dox sucrose agar or in a glucose-phosphate and a salts-mannite medium (Thompson, 1954). Growth failed completely on Sabouraud's glucose or maltose agar at pH 7·1 or 5·1.

Effect of Cystine on the Growth Characteristics

The labile morphology of this organism (v. infra) and the tendency to change its colonial form, or even to die out on repeated subculture, stimulated some preliminary investigation of the factors responsible. For serological investigation it would be useful to have an easily reproducible method for the production of suspensions of coccal forms. The fact that passage on the skin of rabbits rapidly restored the vigour of cultured strains and the restriction of the proliferating organisms in diseased skin to keratinising cells, suggested that sulphur-containing amino-acids might be required in large quantities for the normal complete

TABLE 1

Author	*Organism*	*Milk Clotted and Peptonised*	*Liquefaction of Gelatin*	*Digestion of Inspissated Serum*	*Indole Production*
Bull, 1929	A. dermatonomus	Peptonisation only	+	—	—
Mason and Bekker, 1934	Ditto.	+	+	Softening only	—
Edgar and Keast, 1940	Ditto.	+	+	—	—
Snijders and Jansen, 1956	Ditto.	Acid-clot only	—	—	—
Van Saceghem, 1934	D. congolensis	+	+	—	—
Snijders and Jansen, 1956	S. bovis	Acid-clot only	—	—	—
Chodnik, 1956	Streptomyces sp.	+	+	+	—
Thompson, 1954	Rhizobium sp.	+	—	—	—
Plowright, 1956	Nocardia sp.	+	+ (irregular)	+ (irregular)	—

+ = positive result. — = negative result or not recorded.

"life-cycle", ending in the production of free coccal forms. Observations were therefore made on the effect of incorporation of 0·1 per cent L-cystine in meat-infusion broth, pH 7·4 and in 2·5 per cent nutrient agar prepared from the same base. These media were heavily inoculated with coccal forms and filaments from broth cultures.

In cystine-broth growth regularly took the form of isolated round fluffy balls, which reached about 2 mm. diameter; there was no turbidity. In control broth cultures the usual irregular floccules and marked turbidity were seen. Smears showed that, whilst apparent segmentation of the filaments took place as usual, these did not subsequently break up to yield free cocci. On cystine-agar a tough membranous growth was produced and persisted throughout the life of the culture, smears showing a similar appearance to those in broth. In both solid and liquid media containing cystine it was noted that the majority of the organisms remained intensely Gram-positive, whilst control cultures showed increasing numbers of Gram-negative, "autolytic" cocci (v. infra).

Effect of the Atmosphere of Incubation

No growth occurred in strictly anaerobic conditions. In an atmosphere of 10 per cent CO_2 in air, larger colonies were produced on blood agar plates. The whole of the colonies remained rough with prominent, radiating, surface outgrowths. The transient, aerial mycelium was more abundant and persisted longer whilst a very clear-cut, wide zone of β-haemolysis was produced under these conditions.

Effect of Temperature of Incubation

Growth occurred readily, though more slowly, at 22 to 25°C. On blood-agar colonies reached 0·5 to 1 mm. in 3 to 5 days. The aerial mycelium sometimes appeared to be more profuse and persistent than at 37°C.

Developmental Morphology of the Organism

Repeated subinoculation of cultures on to solid media soon resulted in relatively few colonies being obtained from heavy inocula. Those colonies which did grow showed great irregularity in size and appearance, many of them resembling the "rough" forms described at 72 to 96 hours on blood-agar plates. In 1954, two out of ten isolates investigated produced only rough colonies before the 10th subculture on solid media; at an intermediate stage both rough and smooth type growth was obtained. During the early stages, subculture from single rough or smooth colonies gave rise to both types of colony in each instance. Modifications in the colonial form did not, therefore, seem to imply genetic variations. Smooth colonies were soft, easily removed by a wire loop and readily emulsified in saline. Rough, mature colonies or early "un-

TABLE 2

Author	*Organism*	*Glucose*	*Fructose*	*Lactose*	*Maltose*	*Sucrose*	*Mannite*	*Glycerol*	*Dulcite*	*Dextrin*
Bull, 1929	A. dermatonomus	+	+	—	—	—	—	+	—	—
Mason and Bekker, 1934	Ditto.	+	+	—	+	—	—	—	—	+
Edgar and Keast, 1940	Ditto.	+	—	—	—	—	—	—	—	—
Snijders and Jansen, 1956 ..	Ditto.	+	—	+	+	—	—	—	—	—
Nisbet and Bannatyne, 1955 ..	Ditto.	+	+	—	+	—	—	+	—	—
Van Saceghem, 1934	D. congolensis	+	—	—	+	+	—	—	—	+
Hudson, 1937	A. congolensis	+	+	—	—	—	—	+	—	—
Buck, 1948	Ditto.	+	—	+	—	—	—	+(?)	—	—
Snijders and Jansen, 1956 ..	S. bovis	+	—	+	+	—	—	—	+	—
Thompson, 1954	Rhizobium sp. (?)	+	—	—	—	—	+ (Late)	—	—	—
Plowright, 1957	Nocardia sp.	+	+	—	—	—	—	—	—	—

+ = acid, no gas. — = no acid or gas, or not recorded.

differentiated" growth was tightly adherent to the medium and could not be broken up easily. It was soon realised, from smears, that these "soft" and "hard" forms were a result of varying degrees of subdivision of an initial mycelium, soft colonies consisting largely of coccal forms and broken short filaments, rough colonies including many septate though little-fragmented filaments. From results already given, it was clear that variations in colonial form could be induced by changes in the medium, the temperature or the atmosphere of incubation. It was obvious that individual colonies went through a cycle of development which could be curtailed.

To obtain more precise information, agar impression preparations were made using the method described by Kleineberger (1934). Ox blood agar plates (5 per cent) were heavily seeded with an inoculum consisting largely of coccal forms, using a bent glass rod as a "spreader". Observations began at 5 to 6 hours and continued for 5 to 7 days; the impressions on cover-slips were fixed in Bouin's fluid and stained with dilute Giemsa. The results are detailed below.

5 *to* 6 *hours.* Coccal forms had sprouted to give rise to 1 to 3 intensely-staining basophilic filaments; there were often two, one arising at each pole of an ellipsoidal coccus. Lateral branches were produced by budding and the older, wider parts of the filaments developed a distinct, barred appearance. These transverse bars further divided to give rise to two longitudinal rows of cocci within the parent filament. The radiating outgrowth of branching filaments accounts for the stellate appearance of young colonies; aerial hyphae could not be recognised as morphologically distinct structures.

24 *hours.* Centres of discrete colonies showed dense masses of coccal forms, mixed with broad filaments containing 2 to 4 rows of ellipsoidal cocci. In some instances, dense, rounded clumps of cocci suggested the occurrence of division in three planes. The periphery of the colonies still consisted of filaments extending centrifugally and undergoing branching and septation.

48 *to* 72 *hours.* Peripheral extension had slowed down. In rough colonies long, branching filaments persisted, as heretofore, at the edge, whilst in the smooth ones the mycelium had largely broken down into masses of cocci with short lengths of subdivided filaments.

4 *to* 7 *days.* The majority of the coccal forms at the centre of the colonies were losing their intense basophilia (Gram-positivity) and began to appear as irregular, eosinophilic "envelopes", like small erythrocyte stromata. At the same time certain larger and yeast-like forms became more prominent, especially in persistent lengths of filament; these forms were still intensely basophilic and sometimes showed short, bud-like or filamentous outgrowths. The morphological appearance of the eosinophilic forms and the necessity for heavy subinoculation to produce good subcultures at this stage and later, suggests that the majority of cocci undergo an autolytic process.

In addition to these observations on agar-impression preparations, sections were also prepared of older colonies (14 to 21 days)

growing on serum agar slopes in universal containers. It was found that the surface growth had split up into predominantly free coccal forms, whilst the profuse downgrowths into the medium still consisted of filaments in various stages of segmentation. Heat-fixed, Gram-stained smears from surface colonies or liquid cultures showed, in general, a similar sequence to that noted in agar-impressions, but an additional feature was the presence of Gram-negative sectors in undivided filaments. Such sectors were often to some degree "ballooned" and sometimes contained Gram-positive granules; they were more frequent in cultures incubated in an atmosphere with 10 per cent CO_2 and have also been seen in sections of diseased bovine skin.

There is an obvious need to relate these morphological variations to a life-cycle such as has been described already for Actinomyces (Morris, 1951a), other Nocardia spp. (Morris, 1951b) or Jensenia (Morris, 1952). The large, yeast-like cocci may be "initial cells" and the smaller forms which degenerate could be "A" forms which Morris (1951a) associated with the haploid phase of the life-cycle of Actinomyces and described as becoming Gram-negative.

Observations on Motility

All of many strains of this organism, which we isolated and examined in hanging-drop preparations, produced motile coccal forms. Suspensions from growth on solid media or turbid supernatants from fluid cultures were equally suitable for the demonstration of this property. Repeated subcultures in fluid and on solid media, the latter examined repeatedly and carefully by a stereoscopic microscope, failed to reveal contaminants (Erikson and Porteous, 1955) which could have given rise to errors. The only organism which was encountered at all commonly as a contaminant of primary isolates was a coryneform bacterium which, on subculture to enriched media, rapidly swamped the more slowly growing Nocardia colonies. Motility was also retained after passage on and recovery from the skin of rabbits.

In addition motile cocci were found almost immediately in pure cultures which had been freeze-dried over a period of months and then reconstituted in normal saline. For absolutely conclusive proof the desirability of demonstrating motility in cultures derived from single cell isolates is recognised (Erikson, 1955).

Antibiotic Sensitivity

This was investigated in universal containers of nutrient broth (pH 7·4) containing 10-fold dilutions of the antibiotic. Growth was assessed by visual examination for five days, the inoculum consisting of 3 to 4 drops of a heavy broth culture. Suitable controls were incorporated. The results, (Table 3) indicated that the organism was highly sensitive to all antibiotics used; none of the

latter can, therefore, be used to suppress the growth of contaminants in cultures inoculated with skin-lesion material. At the same time it was found that complete inhibition could also be obtained with as little as 1 g/ml. Penetrane (phenyl-mercuric dinaphthylmethane disulphonate). Its high in vitro activity and its reported ability to penetrate the epidermis would suggest it for controlled observations on the treatment of this group of diseases. The inclusion of 0·1 per cent cystine in the broth did not reverse the inhibitory effect of Penetrane.

TABLE 3

Antibiotic	*Minimal inhibitory concentration (μg/ml. or I.U./ml.)*	
	Strain S140	*Strain S219*
Aureomycin	>5, <0·5	>0·5
Chloramphenicol	>5, <0·5	>0·5
Penicillin..	>1	>10
Streptomycin	>5, <0·5	>5 <1
Terramycin	>0·5	>0·5

DISCUSSION

In 1943, Waksman and Henrici put forward their schema for a classification of the Order Actinomycetales and this is the one which finds most general acceptance today (Bergey, 1948; Erikson, 1949; Anon, 1949; Skinner, Emmons and Tsuchuja, 1951). There are two families; the first, Actinomycetaceae, characterised by the production of a vegetative mycelium which segments into bacillary and coccoid arthrospores, is subdivided into anaerobic or microaerophilic species which are obligate parasites (Actinomyces) and aerobic forms, partially acid-fast or non-acid fast, which are sometimes pathogenic (Nocardia); the second, Streptomycetaceae, has normally an undivided vegetative mycelium, but produces conidia on suitable media. Such forms are rarely, if ever, pathogenic to animals and are subdivided into those which produce chains of spores, Streptomyces, and others where the conidia are formed terminally, either singly or in small clusters on sporophores, Micromonospora.

Since the causal agents of "cutaneous streptothricosis" and allied diseases are consistently found to be aerobic, it follows that such terms as "cutaneous actinomycosis" are, strictly speaking, misnomers if Waksman and Henrici's classification is accepted. Equally well "streptothricosis" is an ill-founded name as there is no valid bacterial genus Streptothrix (Waksman and Henrici, 1943).

In spite of this, Snijders and Jansen (1955) referred to an organism they examined as "Streptothrix bovis". Chodnik (1956) proposed Streptomyces as the proper genus for the agent of bovine cutaneous streptothricosis in West Africa, although he described an unstable mycelium, sometimes absent.

As mentioned in the introduction to this paper, the aerobic filamentous organisms associated with skin diseases of domestic animals have almost invariably been characterised by the production of two or more rows of cocci within branching mycelial filaments. These coccal forms are set free on disintegration of the filament. Since Erikson's (1935) descriptions of the strain N.C.T.C. No. 4572, said to be derived from "cutaneous streptothricosis" of the ox in Nigeria and of *Streptomyces albus* (N.C.T.C. No. 1569), which Erikson (1955a) regarded as very similar, failed to make any reference to this unusual feature, it would appear that the former was not the species usually associated with this disease. The occasional failures to find branching filaments in culture (van Saceghem, 1934; Chodnik, 1956) were probably due to the examination of fixed smears at a stage when the mycelium had already undergone rapid fragmentation.

The rate of mycelial breakdown in Nocardias depends on a great variety of factors including the substrain, the medium, the temperature and atmosphere of incubation and the humidity. Erikson (1954) has dealt with this problem in some detail for a motile soil species, *N. turbata*. It is clearly necessary to distinguish septation from fragmentation, as was found in our experiments on the effects of cystine in the medium. Different colony types, "rough" or "smooth", are due to differences in the rate of liberation of coccal forms (see Morris, 1951 for Actinomyces).

It appeared that cystine prevented fragmentation of the mycelium, rather than favouring it as was expected. Nickerson (1948) brought together evidence from several quarters that cell division, as opposed to growth, in fungi, yeasts and bacteria was an essentially similar process, which depended on an adequate supply of sulphydryl radicles. He found for example that the formation of the yeast phase of *Candida albicans* required an adequate intracellular concentration of —SH groups; the yeast phase would correspond with the coccal one of Nocardia spp. Bullen (1949) showed that the yeast form of *Histoplasma farciminosa* was regularly produced in an atmosphere of 5 to 10 per cent CO_2, whereas, with our organism, such a method of culture resulted in the persistence of rough filamentous colonies. On the other hand, cystine in all these instances favoured the production of the invasive stage, i.e. yeast forms or filamentous forms of the bacteria.

The causal agents of proliferative dermatitis of animals may, in a suitable cultural environment and in parasitised skin tissue, rapidly give rise to free coccal forms. Some of these according to the observations of Jensen (1952), Thompson (1954) and ourselves

are actively motile. Thompson (1954) suggested that the organism he associated with "strawberry foot-rot" should be called a Rhizobium species, not a Nocardia, largely on the grounds of his finding that the coccal forms were sometimes motile, with one or more flagellae. Erikson (1949) referred, with some mistrust, to supposed motile actinomycetes, Nocardia spp., isolated from soil, but subsequently (1954) confirmed motility in a soil species, *N. turbata*. Erikson and Porteous (1955) reported the frequent contamination of strains of pathogenic actinomycetes by other unrelated organisms and Erikson (1955b) asserted that isolates from animal material which exhibited motility had always proved, in her hands, to be mixed cultures. In a discussion of coryneform bacteria, Jensen (1952) noted certain phylogenetic relationships of this group to the actinomycetes and accepted motility as an occasional characteristic of both families. He incidentally remarked that he had examined the organism isolated by Bull (1929) which he considered to be a Nocardia spp., and found it to produce motile coccal forms. (See also Jensen 1953). Bisset and Thompson (1956) re-examined the organism isolated by Thompson (1954) and, on a basis of examination of the septa in filaments and coccal forms, suggested that there might be a relationship with the true cocci, in spite of a superficial resemblance of the motile forms to "swarmers" of Rhizobium.

The aerial mycelium which was observed in this investigation on complex media was usually very short lived (3 to 4 days) and consisted of short straight hyphae, which in impression smears were not differentiated from the rest of the growth. No structures comparable to the coiled sporophores (N.C.T.C. No. 4572) of Erikson (1935) were seen. It appears to be generally accepted that aerial hyphae may occur as rudimentary structures in "soft" Nocardias; they are sterile and wither in 2 to 5 days (Erikson, 1949), having no greater resistance than the rest of the mycelium.

The sugar and other fermentations of this group of organisms are restricted. It is probable that there are minor variations between strains, these differences not being sufficient to serve as a basis for differentiation. The majority of authors have found that there is a definite, though variable, capacity to break down proteins, manifested by the clotting of litmus milk and its subsequent peptonisation and decolorisation, by liquefaction of gelatin and more unusually, by the digestion of inspissated serum. Bull (1929) found that the liquefaction of gelatin only occurred in an alkaline medium (optimum pH 7·6); he also noted fermentation of urea with increasing alkalinity and alkalinisation of brom-cresol-purple milk, a change which he associated with ammonia production. We often detected an ammoniacal smell, especially on opening cultures in sealed vessels. Haemolysis has usually been noted on blood-agar plates, but there has been little further investigation of the phenomenon and especially of the range of haemolytic activity.

We have shown that rabbit and horse erythrocytes were not lysed in agar plates.

It would be particularly interesting to study the ability of these aerobic actinomycetes to digest keratin. In parasitised tissues they are strictly limited to keratinising cells of the superficial and hair-follicle epithelia (Plowright, unpublished), a distribution which bears a very close resemblance to that of ringworm fungi. It is perhaps of significance that certain aerobic actinomycetes isolated from soil do have the ability to decompose keratin, a protein which is said to be resistant to all known enzymes (Nickerson and Williams, 1947). Thus Jensen (1930) isolated two organisms from soil (probably *A. citreus* and Waksman's 145) which could grow in a medium consisting of sand, salts solution and keratin. It is possible that the potential animal pathogens are derived from such soil species. Amongst the ringworm fungi, some species of dermatophytes can exist on keratin only as a source of protein (Daniels, 1953; Bentley, 1953); our experience with maintaining strains of actinomycetes from diseased cattle skin would appear to indicate that none of the common laboratory media do in fact supply such a favourable environment as that provided by keratinising cells.

The conclusion, from the work of others and that reported here, is that these pathogenic aerobic organisms can most accurately be classified as Nocardia spp. It would simplify nomenclature considerably if the associated diseases were called "cutaneous nocardiosis" or "nocardial dermatitis", corresponding to the general use of the term "ringworm" for fungal infections of the skin.

CONCLUSIONS

A brief review is presented of the literature which deals with the nomenclature of skin diseases of domestic animals caused by aerobic filamentous bacteria.

The morphological and cultural characteristics of strains of such organisms isolated from diseased cattle in Nigeria are described. It is suggested that these and similar organisms isolated elsewhere, are "soft" mycelial species of the genus Nocardia.

The Nigerian strains usually produce a scanty, transient, aerial mycelium and give rise to motile coccal forms. They have a limited ability to utilise sugars but show distinct proteolytic properties. The effect of cystine-reinforced media is described.

It is proposed that the terms "nocardial dermatitis" or "cutaneous nocardiosis" should be used for this group of diseases and that such incorrect forms as "cutaneous streptothricosis" should be discontinued.

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THE HISTOPATHOLOGY OF THE BRAIN STEM OF SHEEP AFFECTED WITH NATURAL SCRAPIE

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INTRODUCTION

It is almost generally agreed that scrapie in sheep represents a clinical disease entity, but in spite of over half a century of research and lively interest, no agreement has been reached as to the pathological picture, not to mention the aetiology.

A study of the literature reveals a definite tendency for most investigators to look upon scrapie as a disease of the nervous system (Besnoit and Morel, 1898; Cassirer, 1898; Stockman, 1926; Bertrand, Carré and Lucam, 1937; Brownlee, 1940; Holman and Pattison, 1943; Wilson, Anderson and Smith, 1950). Unfortunately, quite important observations have since been ignored and others have been dismissed sometimes without sufficient evidence. In contrast to the advocates of the nervous nature of scrapie another school of thought has attempted to define scrapie as essentially a disease of skeletal muscle, either due to infestation with *Sarcocystis tenella* (McGowan, 1914) or to a myopathic trait (muscle degeneration of unknown cause) most probably hereditary in origin (Bosanquet, Daniel and Parry, 1956; Parry, 1957).

Recently Zlotnik and Rennie (1957) recorded the presence of vacuolated neurones and certain vascular lesions in the medulla of apparently healthy sheep, changes hitherto considered to be characteristic of scrapie. In a further contribution, Zlotnik (1957 a) compared the incidence of vacuolated neurones in the medulla of apparently healthy sheep with that of animals in advanced clinical scrapie. The results were very striking and showed a definite relationship between the disease and the extent of vacuolation.

The proper evaluation of the above observations is of considerable importance not only for the diagnosis of scrapie but also for the understanding of the pathology of the disease. The present contribution gives details of changes observed in the brain stem of sheep in both advanced and early stages of clinical natural scrapie, and compares them with the lesions seen in apparently unaffected sheep.

MATERIAL AND METHODS

The material consisted of brain stems from 70 sheep affected with natural scrapie and 70 apparently healthy sheep. For the purpose of the paper three groups of animals were chosen (a) 10 sheep in advanced clinical scrapie; (b) 10 sheep showing early symptoms of scrapie; and (c) 10 sheep apparently unaffected and of ages comparable to the diseased animals.

The animals of the two scrapie groups were selected on clinical grounds only. They remained in the Institute under observation for varying periods of time and either died or were slaughtered.

Brains were removed after slaughter and in most cases one thin block of the medulla, and sometimes the cerebellum, was taken for the demonstration of astrocytes; the remainder of the brain was prefixed in 10 per cent. formol saline for 10 days. Cross sections were then cut from the following areas of the brain stem: the diencephalon at the level of the mammillary body; the mesencephalon just posterior to the pineal body; the middle of the pons; the anterior medulla including the trapezoid body (Cut "A" of medulla); both olives (Cut "B" of medulla); the medulla just behind the posterior (inferior) olive (Cut "C" of medulla); the medulla-cord junction.

The blocks, after a further two days fixation in formol saline, were post-fixed either in Bouin's solution or in corrosive formol. Serial paraffin sections, 7 μ in thickness, were stained with haematoxylin and eosin. For the demonstration of Nissl substance paraffin sections were stained with eosin and methylene blue. Myelin was stained as a rule in frozen sections by Spielmeyer's method and occasionally with Sudan Black, but paraffin sections were sometimes also treated according to Weigert-Pal-Ehrhard's technique. The method of Holmes proved excellent for the staining of axis cylinders and intracellular neurofibrils. For the demonstration of fat and degenerating myelin, Sudan IV was used in frozen sections and astrocytes were stained with Cajal's gold chloride and sublimate method. Various cytoplasmic granules and vacuoles were stained with the following techniques:—Heidenhain's iron haematoxylin, Ziehl's carbolic fuchsin, aldehyde fuchsin (Gomôri), Kurnick's methyl green-pyronin, Feulgen's method and periodic acid-Schiff reagent. Acidophilic bodies in the cytoplasm of the neurones were demonstrated by the following method:—stain in haematoxylin (Ehrlich's or Anderson's) for 2 to 3 minutes, then in 1 per cent. aqueous eosin at 60°C. for 10 minutes and differentiate with a saturated solution of tartrazine in cellosolve.

For counting vacuolated neurones 18 consecutive serial sections stained with haematoxylin and eosin were examined from each block. Since three blocks were taken 54 sections in all were scrutinised from each medulla.

OBSERVATIONS

Macroscopic Changes

There were no naked eye changes in scrapied sheep, except for an increase in the cerebrospinal fluid in at least 80 per cent. of the affected animals. The amount of fluid found in the cranial cavity varied from about 5 to 200 ml.; it remained clear and showed no abnormality.

Histological Changes

Neurones

In all the animals of the two scrapie groups there were a number of alternations in the nerve cells. The difference between the more advanced clinical scrapie and the earlier cases was generally only one of degree, but the severity of symptoms was not always followed by more widespread lesions (Tables 1, 2 4 and 5). This was probably due to the fact that it was sometimes difficult to assess the onset of the clinical disease. The changes in the nerve cells

ranged from slight degeneration to necrosis and complete disappearance of the neurones.

Acute swelling of neurones. Affected cells were found throughout the brain stem and were more numerous in the pons and mesencephalon than in the medulla. They were slightly swollen, and the nucleus was slightly distorted and often displaced to the periphery of the cytoplasm. There was a progressive loss of Nissl substance which sometimes began around the nucleus and spread towards the periphery in the form of partial chromatolysis (Fig. 1), while in other cells it started at the periphery and cells could often be seen with Nissl substance surrounding the nucleus only (Fig. 2).

Severe cell change. This type of degeneration was again present throughout the brain stem, but was much more common in the nucleus of the raphé in the medulla, in the reticular formation in both medulla and pons and in the red nucleus of the mesencephalon. Chromatolysis in these cells was almost complete, sometimes only a few irregular masses of granular Nissl substance remaining. The nucleus was completely shrunken and most often found at the periphery of the cell. In other cells the nucleus was very englarged and undergoing liquefaction. In other cells where degeneration had advanced still further the cytoplasm became fenestrated and was either coagulated or had a ragged outline (Figs. 3, 4, 5 and 6). The neurofibrils appeared as aggregated argentophilic masses (Fig. 7).

Pyknotic cells. These cells were quite numerous in all the cases of scrapie and could be found in all parts of the brain stem. They were shrunken, elongated and often had deeply stained, tortuous apical dendrites. In some cells the cytoplasm had completely coagulated and was stained very darkly. In other neurones, very small vacuoles could be seen in the cytoplasm (Figs. 8 and 9).

Necrotic cells. Nerve cells in various stages of disintegration were found in all scrapie animals. They were most numerous in the medulla but were present throughout the brain stem. Sometimes they appeared as single cells, and in other cases the majority of cells in a given area were undergoing necrosis. In most necrotic cells no nucleus could be found; in some, however, the remains of nuclei could still be distinguished, while in others only the nucleolus was visible. The cytoplasm stained deeply with eosin and the cells appeared either as round coagulated bodies, sometimes vacuolated, or were shrunken with ragged edges—sclerotic cells—or were mere shadows of cells. In some advanced cases of scrapie empty spaces often containing distorted cytoplasmic debris were the only evidence of the lost neurones (Figs. 10, 11, 12 and 13).

Multinucleation of nerve cells. These cells were only very rarely seen and when present were found in the medulla-cord junction (Fig. 14).

Ischaemic cell change. These cells were found not only in scrapie cases, but also in non-scrapie animals when the agonal period was

unduly prolonged, and in animals dying from pneumonia. Very severe changes were seen in a case of Jaagsiekte. The cells appeared hyalinized and stained a bright pink with eosin, the cytoplasm being somewhat swollen.

Vacuolation of nerve cells. Vacuolation was present throughout the medulla in all scrapie animals, and in most cases in other parts of the brain stem as well, including the red nucleus of the mesencephalon.

The vacuoles observed in brains from scrapie sheep represented a definite regressive change and were not an artifact due to fixation, dehydration or embedding. They could be demonstrated in frozen sections and in untreated material under dark-field illumination. No specific substance could be detected within the vacuoles and all tests for fat, lipoid, carbohydrate or nucleic acid proved negative, although round or irregular shaped plasmatic bodies were often observed. These bodies stained pink with eosin, but occasionally showed bluish central areas in haematoxylin and eosin preparations. In some cases these bodies represented definite necrotic plasmatic masses (Figs. 15, 25 and 29). It is suggested that the formation of the vacuoles is due either to an oedematous change in the cytoplasm where they represent cavities filled with fluid, or to a degenerative process in the cytoplasm when parts of the cell plasma are undergoing regressive changes or necrosis.

In apparently healthy sheep the vacuoles appeared as small or large single cavities in otherwise normal neurones. In scrapie sheep a great variety of types could be observed. Although a definite classification proved to be difficult the following general types of vacuolation were common :—

1. Single vacuoles, sometimes very small but more often very large, frequently causing extensive ballooning of the cell and displacement of the nucleus but present within a healthy cytoplasm and unaltered Nissl substance. These vacuoles, especially the large ones, are indistinguishable from those seen in healthy sheep (Figs. 16 and 17).

2. Multiple vacuoles, usually very small, within normal cells and most often at the periphery of the cytoplasm (Fig. 18).

3. Single vacuoles, usually large, and very similar to type 1, but in cells undergoing various stages of degeneration (Figs. 19 and 20).

4. Accumulations of small vacuoles within pyknotic or sclerotic cells, either within the actual cell body or in the axone (Fig. 21).

5. Multilocular vacuoles in cells undergoing various stages of chromatolysis. These vacuoles varied in size, the larger ones being formed from a combination of several cavities (Figs. 22—28). A variant is an accumulation of uniform medium sized vacuoles, like punched out holes, filled with necrotic eosinophilic material (Fig. 29).

6. Large single vacuoles or multiple vacuoles in necrotic cells (Figs. 31 and 32).

Types 2 to 6 were found in scrapie sheep only. Types 1, 2, 3 and 5 were the most common, and types 4 and 6 were numerous in

advanced clinical scrapie only. The ratio between the numbers of vacuoles of type 1 to the sum of all the other types varied; in less advanced clinical scrapie the range was 2:1 to 1:4, while in more advanced clinical scrapie the range was 1:2 to 1:10. Cases have been examined where no vacuoles of type 1 could be found, all the unilocular vacuoles belonging to types 3 and 6.

The distribution of vacuoles within the different parts of the brain stem varied from animal to animal. In some single vacuolated cells were widely scattered amongst non-vacuolated cells while in others especially those with advanced clinical scrapie, groups of vacuolated cells were found. It was not uncommon to find vacuoles in all the cells of a nucleus of the medulla (Fig. 30). In each case when cell groups or nuclei showed widespread vacuolation the lesions were symmetrical.

The location of vacuoles in various parts of the brain stem varied to a very large extent from animal to animal. Vacuolated neurones were found in general throughout the brain stem. There was a tendency for vacuolation to be most severe in Cut "C" of the medulla, but in a number of animals vacuoles were more numerous in other parts of the brain stem (cases: S. 18, S. 22, S. 25, C. 233, C. 258, C. 266 and C. 269) while in case C. 268 the pons had nearly as many vacuoles as Cut "C". Tables 1, 2 and 3 give the incidence of vacuoles in various parts of the brain stem with the exception of the diencephalon, since vacuoles were only occasionally found here. The mesencephalon, pons and the medulla-cord junction appear to be no less susceptible to vacuolation than Cuts "A" and "B" of the medulla. This is especially interesting as in unaffected sheep the former parts seldom, if ever, contained vacuoles.

A comparison between the incidence of vacuoles in the medulla in the two scrapie groups and in the healthy group respectively is seen in text figs. 1 and 2. In Fig. 1 the average number of vacuoles per section in Cut "C" only is shown, while in Fig. 2 an average total per section of the medulla is shown. The difference between the incidence in each scrapie group and that in the healthy group is so striking as not to require further comment, but the difference in numbers of vacuoles in animals in advanced scrapie compared with the less advanced disease is not so striking. It can be seen that when Cut "C" of the medulla is examined only two animals with advanced scrapie have more vacuoles than any animal in the non-advanced scrapie group. The difference is, however, more obvious when an average per section of the whole medulla is compared, since four animals have higher numbers than any of the animals in the early scrapie group. To conclude it can be said that as a whole animals in early clinical scrapie have fewer neurones in the medulla affected by vacuolation than animals in advanced stages of the natural disease. It must be pointed out that although figures are

TABLE 1

VACUOLATION OF NEURONES IN ADVANCED SCRAPIE

Serial No.	Total No. of vacuoles in 18 consecutive sections of parts of the Brain stem						Total No. of vacuoles in 54 sections of the Medulla	Average No. of vacuoles per section in the Medulla	Average No. of vacuoles per section in Cut "C" of the Medulla
	Mesencephalon	*Pons*	*Medulla Cut "A"*	*Medulla Cut "B"*	*Medulla Cut "C"*	*Medulla—cord junction*			
S. 3	270	396	288	393	504	90	1185	22	28
S. 18	—	—	1008	180	648	54	1836	34	36
S. 20	54	306	270	288	918	684	1476	27	51
S. 22	—	108	162	972	918	108	2052	38	51
C. 171	—	1134	522	1368	1404	756	3294	61	78
C. 263	1296	324	738	1224	3870	1044	5832	108	215
C. 266	54	179	522	342	396	127	1260	23	22
C. 268	181	1535	450	1440	1584	556	3474	64	88
C. 269	89	305	126	776	412	216	1314	24	23
C. 270	828	1781	917	1135	2214	542	4266	79	123

S. 3, S. 18, S. 20, S. 22, C. 171, C. 263 and C. 270—Suffolks.
C. 266 and C. 268—Swaledales.
C. 269—Cheviot.

TABLE 2

VACUOLATION OF NEURONES IN SHEEP IN NON-ADVANCED SCRAPIE

Serial No.	Total No. of vacuoles in 18 consecutive sections of parts of the Brain stem						Total No. of vacuoles in 54 sections of the Medulla	Average No. of vacuoles per section in the Medulla	Average No. of vacuoles per section in Cut "C" of the Medulla
	Mesencephalon	Pons	Medulla Cut "A"	Medulla Cut "B"	Medulla Cut "C"	Medulla—cord junction			
S. 25	—	—	161	1170	450	199	1781	33	25
S. 41	—	55	57	53	325	—	435	8	18
C. 208	39	1060	103	1337	1530	347	2970	55	85
C. 232	111	234	321	356	763	—	1440	27	42
C. 233	—	75	270	506	415	—	1191	22	23
C. 247	53	73	197	453	1024	416	1674	31	57
C. 258	—	526	57	142	287	70	486	9	16
C. 259	251	234	234	554	634	235	1422	26	35
C. 262	59	58	108	390	420	—	918	17	23
C. 264	216	90	252	215	379	75	846	16	21

S. 25, S. 41, C. 208 and C. 258—Suffolks.
C. 232, C. 233, C. 259 and C. 262—Swaledales.
C. 247—Cheviot.
C. 264—Border Leicester Cross.

given for only 10 animals in each group, these figures are representative of the remainder of the animals so far examined.

Fig. 1

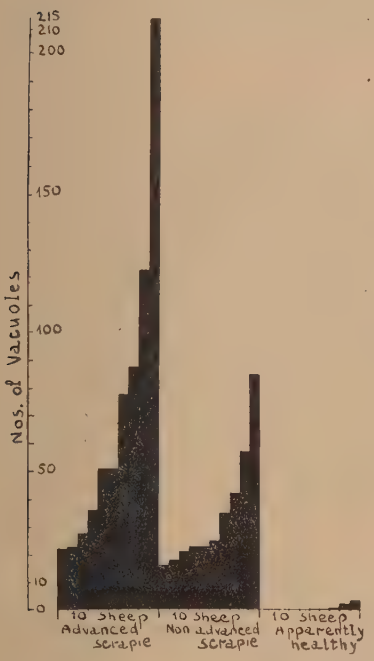
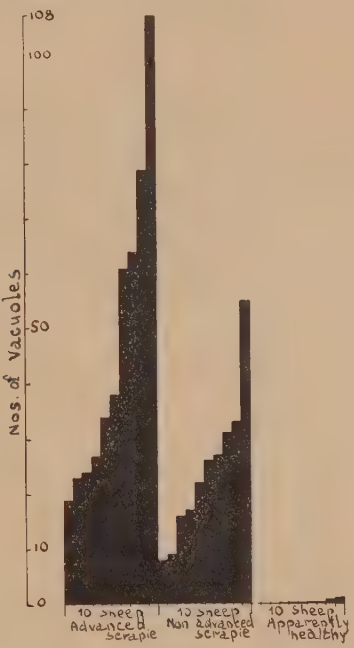


Fig. 2



Text Fig. 1
Average number of vacuoles per section in Cut "C" of the medulla

Text Fig. 2
Average number of vacuoles per section in the whole of the medulla

The distribution of vacuoles in various nuclei of the medulla varied in different animals, but the following nuclei were more commonly and more severely affected than others:—raphé, paramedianus, reticular formation, facial, lateral cuneate and dorsal motor vagus. In the medulla-cord junction the predilection spots for vacuolation were the nucleus ambiguus, pigmentous and formatio reticularis. In the pons, the superior olive, the facial nucleus, the reticular formation and the spinal trigeminal were more often affected than other nuclei. Finally, in the mesencephalon, vacuolated cells could be found anywhere, but on a few occasions the red nucleus appeared severely vacuolated (Fig. 33).

Intracytoplasmic granules. In a search for inclusion bodies various methods have been used, but apart from the two types of granules recorded by the author in a previous communication (1957), no

TABLE 3

VACUOLATION OF NEURONES IN APPARENTLY HEALTHY SHEEP

Serial No.	Total No. of vacuoles in 18 consecutive sections of parts of the Brain stem						Total No. of vacuoles in 54 sections of the Medulla	Average No. of vacuoles per section in the Medulla	Average No. of vacanies per section in Cut "C" of the Medulla
	Mesencephalon	Pons	Medulla Cut "A"	Medulla Cut "B"	Medulla Cut "C"	Medulla—cord junction			
C. 52	—	—	3	—	38	—	41	0·76	2·1
C. 53	—	—	4	1	52	—	57	1·05	2·9
C. 54	—	—	—	—	—	—	—	—	—
C. 55	—	—	6	4	—	—	10	0·18	—
C. 97	—	—	6	4	—	—	10	0·18	—
C. 98	—	—	—	—	3	—	3	0·05	0·17
C. 99	—	—	—	10	4	—	14	0·26	0·22
C. 100	—	—	—	—	—	—	—	—	—
C. 101	—	—	—	—	—	—	—	—	—
C. 102	—	—	—	4	12	—	16	0·3	0·66

C. 52 and C. 53—Border Leicester Cross.
C. 54, C. 55 and C. 97—C. 102—Cheviots.

other bodies could be found. Whatever these bodies represent, whether one is a form of ceroid appearing in ageing cells and the other a secretion, it is clear that no difference could be found between the occurrence of these substances in scrapie sheep and in apparently healthy animals (Figs. 34 and 35).

Vascular Lesions

Perivascular collections of lymphocytes and plasma cells were present in the majority of scrapie and normal sheep, but the severity of the lesions appeared to decline with the progress of clinical symptoms. Nodular infiltrations along capillaries, similar to those described by Zlotnik and Rennie (1957) in healthy sheep, were seen in about 40 per cent. of both scrapie groups. In addition to these two types of lesion, common to both the healthy and scrapie sheep, the following changes (Tables 4, 5 and 6) were observed in the scrapie animals only:— perivascular oedema; loose perivascular proliferation of oligodendroglial perivascular satellites and microglia in which oligodendroglial and microglial cells appeared to form a network at one side of small arteries or round blood vessels (Fig. 36); infiltration of microglial cells within the walls of capillaries (Fig. 37); proliferation and hyalinisation of media of small brain arteries in which these arteries often appeared to form several folds and perivascular haemorrhages were not uncommon (Fig. 38).

Myelin Changes.

No gross tract demyelination was found in any of the scrapie animals although in a few discrete degenerations of myelin sheaths were demonstrated by the Sudan IV method in frozen sections. Perivascular demyelination appeared in 70 per cent. of the advanced scrapie sheep and in 20 per cent. of the less advanced group (Figs. 39 and 40, and Tables 4, 5 and 6). In all the animals showing demyelination, and in the majority of other scrapie sheep where perivascular loss of myelin could not be shown by the ordinary myelin techniques, cells containing fat granules could be demonstrated in the perivascular spaces by means of Sudan IV. The number of compound granular corpuscles varied around each vessel and the number of blood vessels so affected was somewhat higher in the advanced scrapie group.

Glial Changes.

Astrocytes. Proliferation of astrocytes was evident in the medulla in all the scrapie animals. Many nuclei were affected but the most characteristic changes were visible in the inferior olive and in the dorsal motor vagus. In the inferior olive, in some of the advanced cases, the astrocytes formed a very dense network (Fig. 41). In the dorsal motor vagus, only very few astrocytes could be demonstrated by means of Cajal's method in healthy sheep, while in scrapie increased numbers were usually present.

TABLE 4

HISTOLOGICAL CHANGES IN THE BRAIN STEM IN ADVANCED SCRAPIE

Serial No.	Neurone changes			Vascular changes			Perivascular demyelination	Gliosis
	Degeneration	Necrosis	Perineuronal satellitosis	Perivascular infiltrations	Nodular infiltrations	Other vascular changes: (hyalinisation and glial infiltration		
S. 3	+++	++	—	—	—	+	+	++
S. 18	+++	+	+	+	+	++	++	++
S. 20	+++	+++	—	+++	+	+	—	++
S. 22	+++	++	+	+	+	—	++	+++
C. 171	+++	++	—	+	—	—	++	+++
C. 263	++++	++++	+++	+++	—	+	+++	++++
C. 266	++	+	+++	—	—	—	—	+++
C. 268	++	+	+	+	—	—	++	++
C. 269	++	+	+++	+	—	—	—	++
C. 270	++++	++++	+++	—	+	++	+	++++

\+ slight.
\+ + moderate.
\+ + + severe.
\+ + + + very severe.

TABLE 5

HISTOLOGICAL CHANGES IN THE BRAIN STEM IN NON ADVANCED SCRAPIE

Serial No.	Neurone changes			Vascular changes			Perivascular demyelination	Gliosis
	Degeneration	Necrosis	Perineuronal satellitosis	Perivascular infiltrations	Nodular infiltrations	Other vascular changes: (hyalinisation and glial infiltration)		
S. 25	++	+	—	+++	+	—	++	+
S. 41	+	+	++++	—	+	—	—	++
C. 208	+	+	+	++	+	++	—	+
C. 232	++	+	+	++	+	—	+	+
C. 233	+++	+++	—	—	—	++	—	+
C. 247	+	+	++	—	—	+++	—	+
C. 258	+++	+++	+	++	—	—	—	++
C. 259	++	+	+	++	—	—	—	+
C. 262	+	+	+	++++	—	++	—	++
C. 264	++	+	—	++	—	—	—	+

\+ slight.
++ moderate.
+++ severe.
++++ very severe.

TABLE 6

HISTOLOGICAL CHANGES IN THE BRAIN STEM OF APPARENTLY HEALTHY SHEEP

Serial No.	*Neurone changes*			*Vascular changes*			*Perivascular demyelination*	*Gliosis*
	Degeneration	*Necrosis*	*Perineuronal satellitosis*	*Perivascular infiltrations*	*Nodular infiltrations*	*Other vascular changes*		
C. 52	—	—	+	+++	+	—	—	—
C. 53	—	—	+	++	+	—	—	—
C. 54	—	—	—	++	—	—	—	—
C. 55	—	—	—	+++	—	—	—	—
C. 97	—	—	—	+++	—	—	—	—
C. 98	—	—	—	+++	—	—	—	—
C. 99	—	—	+	+	—	—	—	+
C. 100	—	—	—	+	+	—	—	—
C. 101	—	—	—	—	—	—	—	—
C. 102	—	—	+	+++	—	—	—	+

\+ slight.
++ moderate.
+++ severe
++++ very severe.

Oligodendroglia and microglia. In most scrapie animals oligodendrogliosis or satellitosis was pronounced. In many degenerating neurones the perineuronal satellites were embedded inside the actual cytoplasm and exhibited neuronophagia (Fig. 42). In the more advanced cases of scrapie ologodendroglial cells together with microglial cells formed perineuronal glial rosettes, perivascular glial knots and focal infiltrations in the grey matter, or very diffuse infiltrations especially within the various nuclei of the medulla (Figs. 43 and 44).

Cerebellum. In addition to the brain stem the cerebellum was often examined and in most cases, apart from occasional vacuolation and disappearance of isolated Purkinje cells, no lesions could be detected. In a few cases, however, increased proliferation of astrocytes in the granular layer was obvious and the cells of the granular layer penetrated the Purkinje layer and about half way into the molecular layer. In other cases a severe oedema and vacuolation of the granular and Purkinje layers was observed. A point of interest is that only one folium in a given area was so affected while adjacent folia showed little abnormality (Fig. 45).

DISCUSSION

Until recently there was a general consensus of opinion that cerebral changes could always be found in sheep affected with natural scrapie. Besnoit and Morel (1898) described disintegration of the Nissl substance and vacuolation of the neurones in the medulla, at the same time pointing out that only a few cells were thus affected. Stockman (1926) found in almost every case of scrapie vacuolated neurones in the medulla and the cord, and degeneration of single myelin fibres. He considered, however, that the granules observed in many neurones were of greater importance than any other lesions, and compared these intracytoplasmic bodies with the Negri bodies of rabies. In 1937, Bertrand, Carré and Lucam in their work on scrapie described the following lesions in the central nervous system of about 20 animals destroyed in the last stages of clinical scrape :—vacuolation with ballooning of nerve cells, degeneration and liquefaction of neurones in the medulla, cellular atrophy, severe cell change, cloudy swelling of neurones, severe changes in the neurofibrillary apparatus, satellitosis and true gliosis of the grey matter in the medulla, cord and cerebellum, and finally only very rarely perivascular infiltrations. These lesions were also recorded later by Lucam, Béchade and Saurat (1950) in a further outbreak of scrapie where in addition to degenerative changes in the neurones discrete perivascular cuffings were also present. Brownlee (1940) could not confirm the results of Bertrand, Carré and Lucam. Apart from large vacuoles in otherwise healthy neurones of the medulla and cord, Brownlee found no other lesions in the central nervous system of sheep affected with natural scrapie.

In Brownlee's opinion vacuoles, similar to those of scrapie sheep, could not be demonstrated in sheep suffering from other diseases, although very occasionally vacuolated cells were seen in louping-ill; these, however, were much smaller in size and occurred in necrotic cells only. The work of Holman and Pattison (1943) seems to throw further light on the problem of lesions in the brain stem of scrapie sheep. According to these writers vacuoles in the medulla were present in all 75 clinical cases of scrapie and were easily found in all but 3 cases. Vacuolated neurones were also seen in the pons and mid-brain. Not all the vacuoles, however, appeared in otherwise healthy cells as suggested by Brownlee, but also in many cases in severely degenerated neurones. The largest concentration of vacuoles was found in the medulla just anterior to the calamus scriptorius, that is the anterior part of Cut "C" of the present investigation. Eosinophilic granules within some of the vacuoles were also described by Holman and Pattison. It thus appears that vacuolation of neurones is, if not specific, at least significant for natural scrapie. Some workers demonstrated other lesions in the central nervous system, such as degeneration of neurones, degeneration of single myelin sheaths, gliosis and rarely perivascular infiltrations. Confirmation of these results came from the work of Wilson, Anderson and Smith (1950) who found vacuolation of neurones in the medulla, microglial proliferation in the brain stem and perivascular cuffings in sheep in which scrapie had been induced experimentally, thus proving that the experimental disease was similar to, if not identical with, natural scrapie.

All these consistent claims of lesions in the central nervous system were, however, denied recently by Bosanquet, Daniel and Parry (1956). These writers, although admitting that they have not completed their examination of the central nervous system, stated that no lesions could be demonstrated in the brain and that vacuoles were only occasionally seen in the medulla and spinal cord of scrapie sheep. They suggested that their appearance was of little significance because they found them in normal sheep and in humans affected by various diseases. In a further communication, Parry (1957) claimed that a preliminary examination of the nervous system did not reveal any conspicuous changes apart from occasional vacuoles in the larger neurones of the brain and cord. He did not state how many animals were examined, or in what stages of scrapie or what parts of the central nervous system were studied. The work of Bosanquet, *et al.* (1956) and Parry (1957) was followed by a contribution from Palmer (1957) who, referring to the claim of Bosanquet, *et al* that vacuolated neurones are seen in non-scrapie animals, described eosinophilic bodies within the vacuoles and in the cytoplasm of neurones in the medulla from scrapie sheep only. This observation was contested by the present writer (1957) because these bodies were found by him in both scrapie and apparently healthy sheep.

The results of the present study do not support the views of Bosanquet, *et al* (1956) and Parry (1957) since lesions were always found in the brain stem of scrapie sheep. Vacuolation was usually severe, far from occasional and not confined to larger neurones but present as a rule in medium-sized and small neurones; the very large neurones, on the other hand, were less often affected. The present investigation confirms the findings of Bertrand, Carré and Lucam (1937), Holman and Pattison (1953) and Pattison (1957). Vacuolation appears to be a very characteristic lesion in scrapie and comparison of the numbers of vacuolated neurones between healthy and affected sheep is in itself convincing. Further evidence is shown that degeneration of neurones is a definite feature in scrapie and so far no case of clinical scrapie has been seen without it. Degenerated cells can further be vacuolated and various types of vacuoles have been described. It is difficult to determine whether vacuolation is superimposed on degeneration, as vacuoles can be seen in cells undergoing regressive changes and also in cells otherwise normal; on the other hand severely degenerated and necrotic cells were seen without vacuolation. According to Courville (1950) vacuolation occurs in nerve cells under many different circumstances and in many conditions e.g. amyotrophic lateral sclerosis, and vacuoles may represent either real cavities filled with fluid or they may be made up of products of degeneration. It is obvious from the present study that in scrapie vacuoles may be formed in both ways.

The distribution of vacuoles varied in different animals, and although in most sheep the highest concentration was in the medulla just anterior to the calamus scriptorius, or the anterior half of Cut "C", vacuoles were usually numerous in other parts of the brain stem, especially in the pons. Vacuoles and various degrees of degeneration were present in the mesencephalon in 70 per cent. of advanced scrapie animals, and in 60 per cent. of non-advanced clinical sheep. The red nucleus, the seat of constant lesions in swayback (Innes and Shearer, 1940), was occasionally affected with either chromatolysis or vacuolation or both.

Apart from vacuolation and degeneration of neurones, astrocytic proliferation was a constant lesion in scrapie sheep, which is in agreement with the findings of Bertrand, Carré and Lucam (1937). In the few cases where gliosis was observed in healthy sheep the cells were a mixture of microglia and oligodendroglia.

Perivascular infiltrations were only rarely found in advanced natural scrapie by Bertrand, Carré and Lucam (1937), while Wilson, Anderson and Smith (1950) described a wide distribution of these lesions in experimental scrapie. The work of Zlotnik and Rennie (1957) has shown that these lesions, as well as some focal microglial infiltrations, are seen in apparently healthy animals. It may be significant that in the present study perivascular cuffings were severe in the non-advanced scrapie group, but of little importance in the advanced cases; this is in full agreement with the c

findings of Bertrand, Carré and Lucam. It is not possible to decide whether the occurrence of these lesions in experimental animals and in early clinical scrapie is due to an infection of the brain. Perivascular cuffings are not an absolute indication of infection for, according to Courville (1950), accumulations of leucocytes around vessels may be due to such causes as vascular disease, degenerative conditions and neoplasms. In these instances the cuffing is the result of irritation by products of degeneration. Furthermore, vascular changes of even greater severity than those in both scrapie groups were seen in healthy sheep where apparently no infection was present. No suitable explanation can be put forward for the presence of perivascular infiltrations in otherwise healthy sheep. Their occurrence is especially interesting since the incidence of perivascular leucocytic infiltrations seemed to decline with the advancement of the disease.

In a previous paper (Zlotnik and Rennie, 1957) three possible explanations were advanced for the occurrence of vacuolated neurones in apparently healthy sheep, and in view of the present findings these suggestions need to be reconsidered. It is possible that vacuoles present in otherwise normal neurones in non-scrapie animals indicate that such animals were in the incubation period of scrapie, or were affected with a non-clinical form of the disease. On the other hand, the fact that some neurones in non-scrapie animals might react to a slight oedematous change in the brain or to an unknown toxic factor by vacuolation cannot be overlooked. Whatever the cause of vacuolation in non-scrapie animals, the number of vacuoles is always less than in animals with clinical scrapie. So far the numbers of vacuoles present in 54 serial sections from three levels of the medulla of non-scrapie animals have not exceeded 100 and were usually much less, while in scrapie animals the corresponding figure was always over 400 and more often well over 1000, especially in advanced scrapie. The large numerical difference in the incidence of vacuoles in scrapie and non-scrapie animals shows that vacuolation is directly connected with scrapie. No other disease of sheep is known to stimulate to such an extent the vacuolation of neurones in the brain stem.

CONCLUSIONS

Various degrees of vacuolation and degeneration of neurones were observed in the brain stem in scrapie sheep.

Degeneration and vacuolation of neurones and astrocytic proliferation in the gray matter were the most constant lesions in the brain stem in both early and advanced stages of scrapie.

Various glial changes and vascular lesions are reported.

Perivascular demyelination was of common occurrence in advanced scrapie.

The numerical difference in the incidence of vacuolated neurones between the scrapie animals and apparently healthy

sheep is discussed. The number of vacuoles in sheep affected with natural scrapie is much higher than in apparently healthy sheep and there is a relationship between vacuolation and the clinical stage of the disease.

ACKNOWLEDGMENTS

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LEGENDS TO ILLUSTRATIONS

Figs. 1 and 2. Acute swelling of neurones. Eosin-methylene blue × 240.

Figs. 3 and 4. Severe chromatolysis of neurones. Eosin-methylene blue × 240.

Fig. 5. Severe cell change. Eosin-methylene blue × 240.

Fig. 6. Complete chromatolysis in a neurone in the red nucleus. Eosin-methylene blue × 240.

Fig. 7. Neurofibrils in severe cell change. Holmes method × 600.

Figs. 8 and 9. Pyknotic cells. H. & E. × 240.

Figs. 10 and 11. Neurones undergoing necrosis. H. & E. × 240.

Fig. 12. Neurones in various stages of degeneration (note empty space after a lost neurone). H. & E. × 180.

Fig. 13. Necrotic neurones. Eosin-methylene blue × 100.

Fig. 14. Multinucleated neurone. H. & E. × 240.

Fig. 15. Vacuole containing large necrotic mass. H. & E. × 240.

Figs. 16 and 17. Vacuoles in otherwise healthy cells (Type 1). H. & E. × 240.

Fig. 18. Multiple vacuoles in an apparently normal cell (Type 2). H. & E. × 240.

Fig. 19. Single vacuole in cell affected with peripheral chromatolysis (Type 3). Eosin-methylene blue × 240.

Fig. 20. Single vacuoles in pyknotic cells (Type 3). H. & E. × 240.

Fig. 21. Multiple vacuole in a pyknotic cell (Type 4). H. & E. × 240.

Figs. 22 to 24. Multiple vacuoles (Type 5). Eosin-methylene blue × 240.

Fig. 25. Multiple vacuoles (Type 5) and single vacuole (Type 3) both containing eosinophilic bodies. H. & E. × 100.

Figs. 26 to 29. Multiple vacuoles (Type 5). Eosin-methylene blue × 240.

Fig. 30. Severe vacuolation of the lateral cuneate nucleus of the medulla. H. & E. × 100.

Fig. 31. Multiple vacuoles in a necrotic cell (Type 6). H. & E. × 240.

Fig. 32. Single vacuole in a cell undergoing necrosis. H. & E. × 240.

Fig. 33. Vacuolation of the neurones in the red nucleus. H. & E. × 100.

Fig. 34. Ceroid-like granules in neurones of the medulla. Aldehyde-fuchsin (Gomori's) × 240.

Fig. 35. Spherical eosinophilic bodies in a neurone of the medulla. Haematoxylin, eosin and tartrazine × 240.

Fig. 36. Loose perivascular mixed glial proliferation. H. & E. × 100.

Fig. 37. Microglial infiltration in the wall of a capillary. H. & E. × 240.

Fig. 38. Hyalinization of media of a small artery and perivascular haemorrhage. Eosin-methylene blue × 240.

Fig. 39. Perivascular demyelination in the medulla. Spielmeyer × 100.

Fig. 40. Perivascular demyelination and gitter cells. Iron-alum haematoxylin × 100.

Fig. 41. Astrocytic proliferation in the inferior olive. Cajal × 100.

Fig. 42. Neuronophagia. Eosin-methylene blue × 240.

Fig. 43. Perineural glial infiltration. H. & E. × 240.

Fig. 44. Focal glial infiltration. H. & E. × 100.

Fig. 45. Oedema and vacuolation in the cerebellum. H. & E. × 25.

Plate I

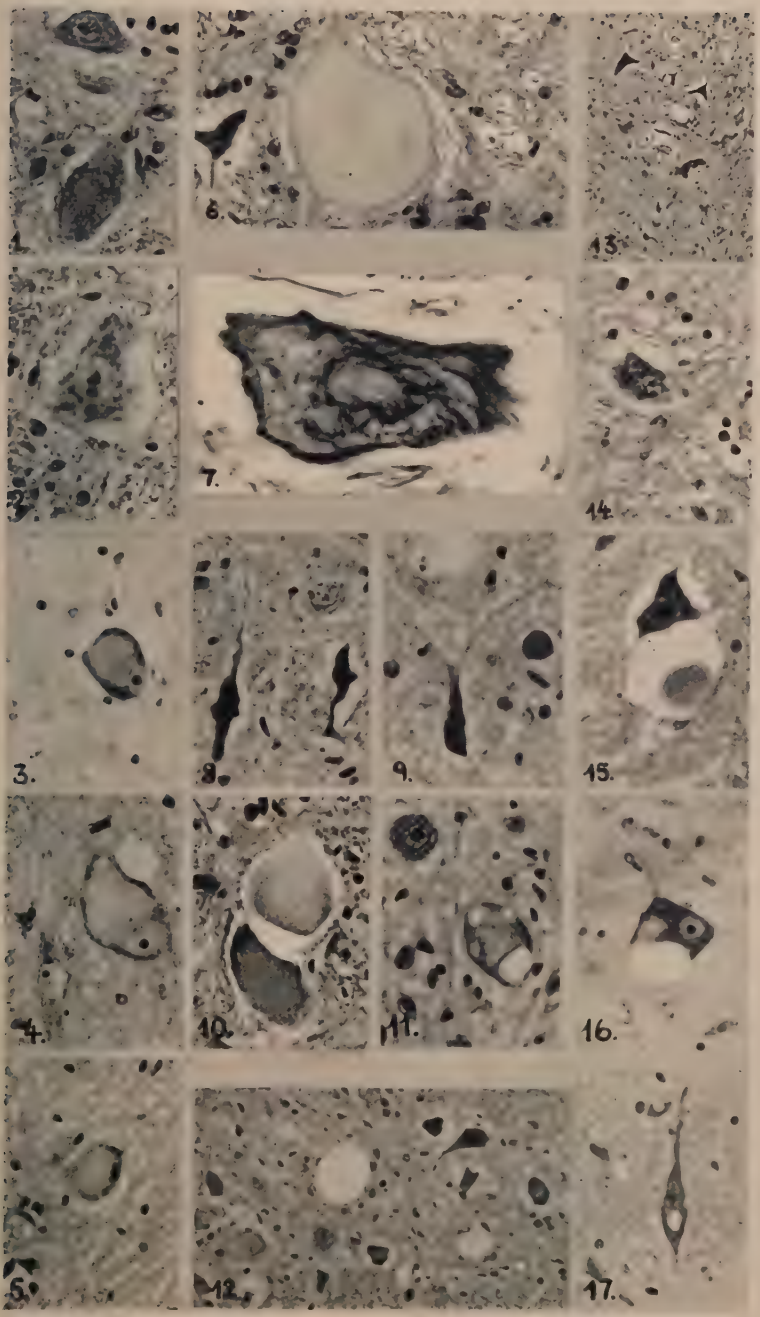

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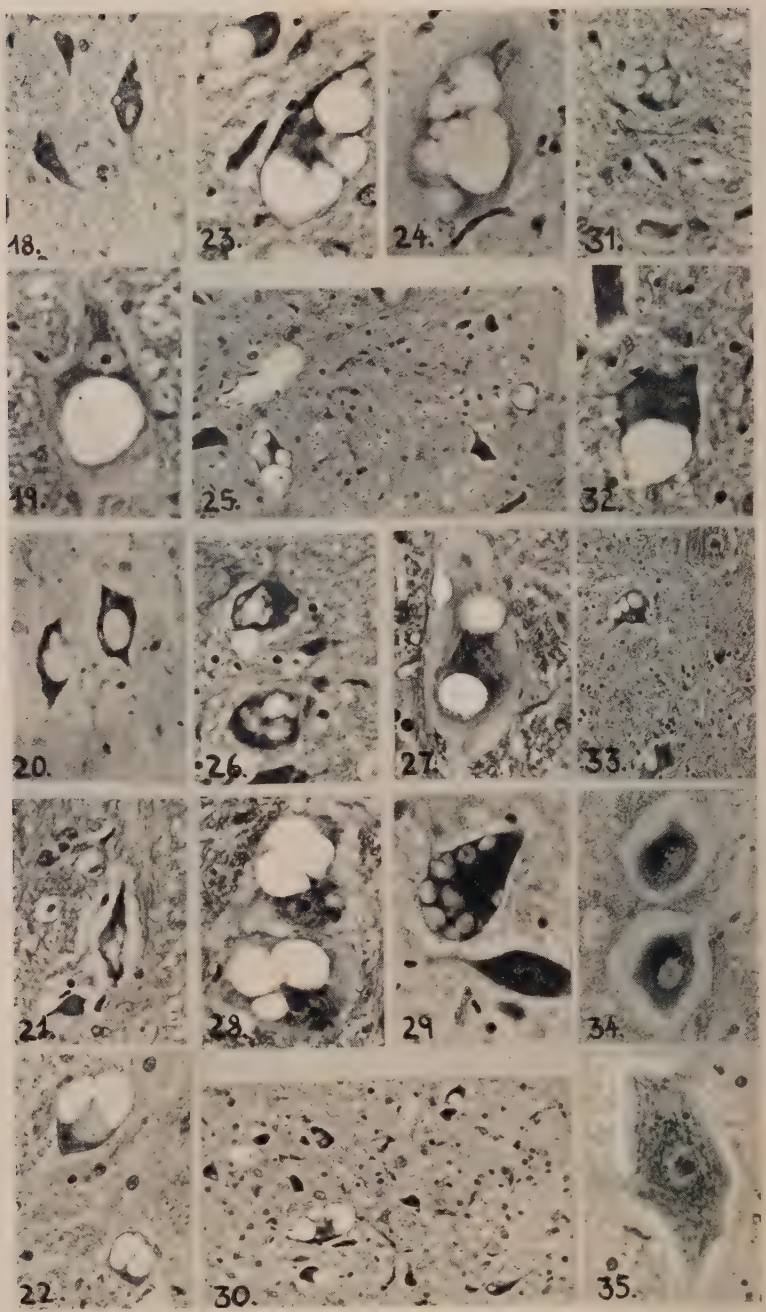
Plate II



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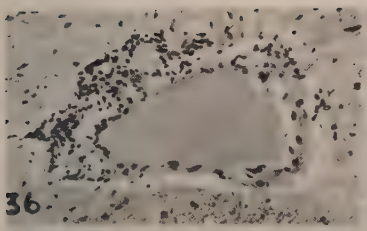

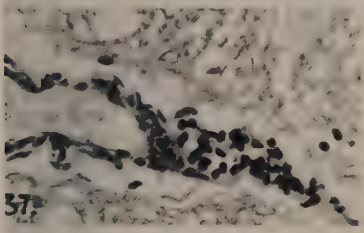

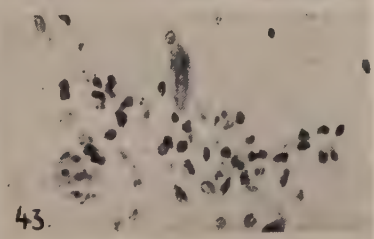

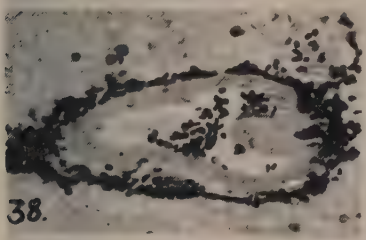

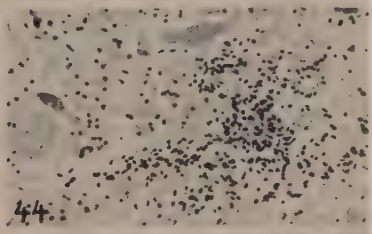

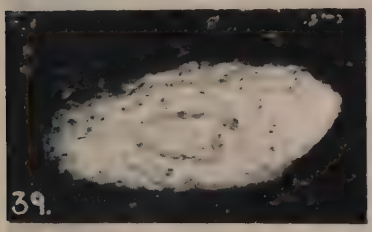

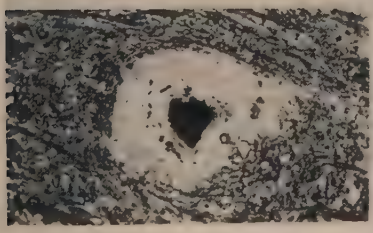

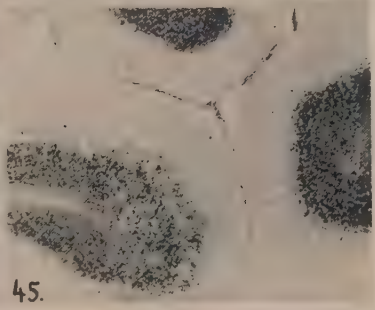

PATHOLOGICAL AND PHARMACOLOGICAL EXAMINATION OF A CANINE TUMOUR OF THE ADRENAL MEDULLA

By

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INTRODUCTION

This paper describes the pathological and pharmacological examination of a phaeochromocytoma of the right adrenal gland of an Old English sheepdog 14 years of age. Recently two tumours of this type have been reported, one by Tamaschke (1955) in a nine-year-old Doberman bitch and one by Müller, Werle and Sell (1955) in a seven-year-old male hound. These latter authors showed that noradrenaline was present in the tumour tissue. Both groups of workers summarized the incidence of hyperplasia and neoplasia of the adrenal glands of dogs and indicated that phaeochromocytomas are rare in this species.

MATERIALS AND METHODS

The autopsy was carried out immediately after death of the dog. Blocks of tissues (approximately 1 cm. square and 0.25 to 0.5 cm. thick) were placed in fixative within one hour of death. Those fixed in 10 per cent formol-saline were processed either to frozen sections and stained with Sudan IV, or to paraffin sections and stained with Anderson's haematoxylin and eosin. The special techniques employed in the adrenal glands are described in the text under the heading 'histological examination'.

For the pharmacological work, 15.95 g. of tumour were placed in 15 ml. of 0.01 N-HCl about two hours after the destruction of the dog. This extract was concentrated as previously described (Head and West, 1955) and assayed for its adrenaline and noradrenaline content by paper chromatography and biological methods (Shepherd and West, 1951).

For the paper chromatographic analysis of the extract for other possible precursors of adrenaline, a further 20-fold concentration of the extract was made (Head and West, 1955).

RESULTS

Clinical History

In May 1953, when the dog was 12 years old, the testicles were removed because they were enlarged. Macroscopic and histological examination showed that one testicle (100 g. in weight and measuring 6 cm. in diameter) was completely replaced by a seminoma; the

other (25 g. in weight and measuring 3 × 2 × 1 cm.) showed an early multifocal seminoma formation. In August 1955, the dog was again presented for examination with the history that it had been quite well since operation, but during the last two weeks it had developed dyspnoea which had become progressively worse. Anorexia and lethargy had been noted, and on auscultation of the heart, valvular murmurs were heard. The dog was destroyed by an intravenous injection of Pentothal Sodium.

Post-mortem Examination

The carcase was well developed but very fat and there was an encapsulated mass of fat (measuring 5 × 4 × 2 cm.) lying under the cutaneous muscle over the sternum. The heart showed left ventricular hypertrophy; both atrio-ventricular valves were thickened and distorted by oedematous fibrous thickenings, especially marked on the left side. The liver was congested and there were numerous hyperplastic nodules in all lobes. The gall bladder contained a semi-solid, greenish-black calculus. The prostate gland was small for the size of the dog, each lobe measuring 3 × 2 × 2 cm. Some pancreatic lobules were slightly larger and paler than others.

The left adrenal gland weighed 1 g. and was L-shaped, each limb being 1 cm. long × 1 cm. wide × 0.5 cm. thick; on the cut surface, numerous yellow nodules of cortical tissue (up to 0.25 cm. in diameter) extended into the red medullary tissue. The right adrenal gland weighed 25 g. and consisted of a sphere, 3 cm. in diameter, originating at the posterior pole from which extended a protuberance measuring 1 × 1.5 × 1 cm., whilst anteriorly there was a papillary cellular mass measuring 2.5 × 2 × 2 cm. bursting from the cortex. On the cut surface, this anterior mass was composed of white to pink soft cellular tissue with a thin connective tissue capsule separating it from the surrounding fat. Similar white to pink tumour tissue completely replaced the medulla of the central spherical region and the posterior protuberance, but in these regions it showed a faint lobulation and small punctiform haemorrhages. Unlike the anterior mass, it was surrounded by a sheet of yellow cortical tissue, 0.1 cm thick.

Histological Examination

Although many organs were examined, only those showing abnormalities are described. The acquired lipomatoid mass in the sternal region was encapsulated, adult adipose tissue. The liver showed centrilobular congestion and fatty change, the latter being most marked in the regions of nodular hyperplasia. Throughout the liver, the von Kupffer cells contained a considerable amount of haemosiderin. The kidney showed focal cortical interstitial aggregations of plasma cells and lymphocytes with some interstitial fibrosis at the corticomedullary junction where the tubules (loops of Henle) showed fatty change. The acini of the prostate gland were lined by one or two layers of cuboidal epithelium and were separated from one another by an increased amount of fibrous tissue. The cells in the large pale lobules of the pancreas were larger and paler

staining than normal exocrine cells. The presence of nodular hyperplasia of the cortex of the left adrenal gland was seen to extend into both medulla and capsule (Fig. 1).

The anterior pole of the right adrenal gland showed only a thin connective tissue capsule while the remainder of the gland had a thin layer of cortical tissue peripherally with no evidence of zonary formation. In the connective tissue capsule of the posterior pole, there were occasional foci of disorderly cortical cells with a few medullary cells centrally (Fig. 2). There was a moderately distinct junction between the cortex and the medullary tumour, which had a similar structure in its anterior and posterior poles. The tumour was composed of irregular masses of cells formed into lobules by connective tissue and possessing blood vessels of similar abundance to those in the left adrenal medulla (compare Figs. 1 and 2). However, some of the blood vessels were in the form of large cavernous spaces (Fig. 2). There were some spaces between the tumour cells, due possibly to a fixation artefact. Occasional areas, irregular in shape, showed coagulative necrosis with ghost outlines of cells and in this region the peripheral nuclei showed pyknosis and karyolysis. The cytoplasm of the tumour cells was generally somewhat smaller than that of the cells of the normal left adrenal medulla (compare Figs. 5 and 6), though occasionally it was very large. With silver impregnation (Gordon and Sweet's method), the left adrenal medulla was formed into 'packets' of 10 to 12 cells by a well defined reticulin network, whereas there was less reticulin in the tumour of the right adrenal medulla and the cell 'packets' were much larger (compare Figs. 3 and 4).

Although some of the cells in both left adrenal medulla and the right adrenal medullary tumour showed vacuolation in the cytoplasm, only the cells round the areas of necrosis in the tumour contain sudanophilic material in frozen sections.

Tissues fixed in formalin, processed to paraffin wax and stained H. & E., showed faint purple granulation in both left and right adrenal medullary cells. When the ferricyanide reduction test was employed (Schmorl's reaction, quoted Pearse, 1953), this purple granulation became more distinct and some cells showed dark blue granules (Figs. 5 and 6). With the P.A.S. technique, only occasional cells, probably young mast cells, gave a positive reaction. Formalin-fixed sections treated with 3 per cent potassium dichromate for three hours before being stained H. & E. showed no difference in reaction from straight H. & E. staining.

Blocks placed in 3 per cent potassium dichromate for three days and processed by the frozen section technique showed a diffuse brown coloration of similar intensity in both left adrenal medulla and right adrenal medullary tumour, but no granules could be resolved, even under oil immersion examination. When such blocks were processed to paraffin wax, the brown coloration was less marked. Chromated paraffin wax sections with Schmorl's reaction

gave a similar though rather muddy reaction and granules were again indistinct. The P.A.S. technique in chromated paraffin wax sections gave negative results.

Identical results have been obtained using 3 per cent potassium iodate in place of potassium dichromate, though the intensity of coloration was slightly less marked.

Pharmacological Examination

The assay on the blood pressure and nictitating membrane of the spinal cat showed that the total catechol amine content of the tumour tissue was 0.95 mg/g. fresh tissue. This figure is well below that found by Shepherd and West (1951) in the normal dog adrenal medulla, (1.50 mg/g. fresh tissue). Whereas in the normal medulla, noradrenaline constitutes 27 per cent of the total, in this tumour the figure was 99 per cent of the total. A similar though less marked alteration in the relative noradrenaline content was noted by Shepherd and West (1951) when they compared the adrenal glands of adult dogs with those of puppies.

Despite the 20-fold concentration of amine content in the tumour extract, paper chromatographic analysis showed that only adrenaline and noradrenaline were present. Hydroxytyramine, dihydroxyphenylalanine, dihydroxyphenylserine, lactyl-adrenaline, lactyl-noradrenaline, *p*-norsynephrine, *m*-norsynephrine and tyramine were not detected.

DISCUSSION

The tumour in the right adrenal gland of this dog was well differentiated and resembled the tissue of the left adrenal medulla. It showed no evidence of infiltrative growth, suggesting therefore that it was a benign tumour of the chromaffin cells of the adrenal medulla (a phaeochromocytoma). Yet the cells were immature and their grossly raised relative noradrenaline content resembled that found in foetal adrenal glands. The methylating process in the tumour of the dog was retarded, and this may mean that the adrenaline-producing cells were not functioning or may not have been present in the tumour tissue. The present case therefore was similar to that of a bovine adrenal medullary tumour reported previously (Head and West, 1955).

Müller, Werle and Sell (1955) explained the sudden development of the severe fatal dyspnoea in their case of canine phaeochromocytoma as being due to congestion and oedema of the lungs resulting from the discharge of pressor substances from the tumour in short bursts. The paroxysmal hypertension produced degenerative changes and cellular infiltration in the heart muscle, with alterations in the calibre of the smaller blood vessels both in the heart and in the lungs. The clinical history in our case was of progressive, but not sudden and fatal, dyspnoea, and no major changes in the blood vessels of the heart and lungs could be found.

These differences could be explained on the grounds that our case was destroyed and not left to die.

In the present case, there was the complicating factor of the presence of lesions in the atrio-ventricular valves of the heart. The valvular incompetence thus produced possibly led to the chronic venous congestion in the liver and subsequent nodular hyperplasia. As has been mentioned by Detwieler (1952), chronic valvular endocarditis is quite common in old dogs. Shouse and Meier (1956) suggest that these chronic valvular lesions, which they consider to be the most common cause of cardiac insufficiency in dogs, might be the end result of acute vegetative endocarditis.

Although the amount of adrenal medullary tissue in the present case was at least 25 times that normally present, the total catechol activity per gram of tumour was lower than that normally found in dog adrenal glands. This result was quite unlike that found in cases of phaeochromocytoma reported in the literature for other species (West, 1955). We have no evidence to suggest that the tumour was continually secreting large quantities of noradrenaline, but had it been doing so the noradrenaline with its special ability of maintaining normal blood pressure levels might have compensated for the effects of the heart valve lesions.

There was no reason to suppose that the lesions in the kidney were related to the tumour in the adrenal, since these lesions were similar to those which develop following *Leptospira canicola* infection, a common disease in the district where the dog was housed (McIntyre and Montgomery, 1952). Shouse and Meier (1956) have suggested that such lesions may be the result of healed infarctions arising from emboli liberated from acute vegetative valvular endocarditis. Since we did not perform serological examination for leptospiral agglutinins in this case, we cannot offer any constructive comment on this.

One might be tempted to suspect that the nodular hyperplasia of the left adrenal cortex was compensatory for the compression and destruction of the right adrenal cortex by the tumour. As Tamaschke (1955) has pointed out, however, such nodules are common in the older age groups of dogs. In our experience, they are frequently found with multiglandular hormonal imbalance; in this case the seminoma and nodular hyperplasia of the pancreas could be so linked. Acquired lipomatoid masses are also common in such cases. Cohrs (1927) comments on the frequency of multiple primary tumours in dogs, but in none of his cases and in none of our previous cases has the adrenal medulla been involved. It would seem unlikely therefore, from this evidence and from the rarity of phaeochromocytoma in dogs, that the tumour in this case was part of the multiglandular syndrome. The atrophy of the prostate was probably due to castration two years before the animal's destruction. In our experience biliary calculi are rarely

seen in dogs, and the significance of the one seen in this case is unknown.

Our results with the various histochemical tests closely resemble those described by Pearse (1953). It is of interest that we agree with Pearse that chromaffin granules in dog adrenal glands do not give a positive P.A.S. reaction and cannot be demonstrated with dichromate subsequent to fixation in formalin.

CONCLUSIONS

The pathology of a phaeochromocytoma of the right adrenal gland of a fourteen-year-old male Old English Sheepdog is described. Pharmacological analysis of the tumour showed that 99 per cent of the catechol amine fraction consisted of noradrenaline. The composition of the tumour thus resembles that of the foetal adrenal medulla.

ACKNOWLEDGMENTS

The writers wish to thank the following members of the staff of the Royal (Dick) School of Veterinary Studies for their help; Mr. A. R. Withers, B.Sc., M.R.C.V.S., Department of Clinical Veterinary Medicine and Surgery whose case this was; Mr. I. S. Beattie, B.Sc., M.R.C.V.S., Department of Veterinary Pathology, for help and advice in the preparation of this paper; Mr. R. C. James, F.I.M.L.T. and Mr. J. Heeps, A.I.M.L.T. for technical assistance and photography.

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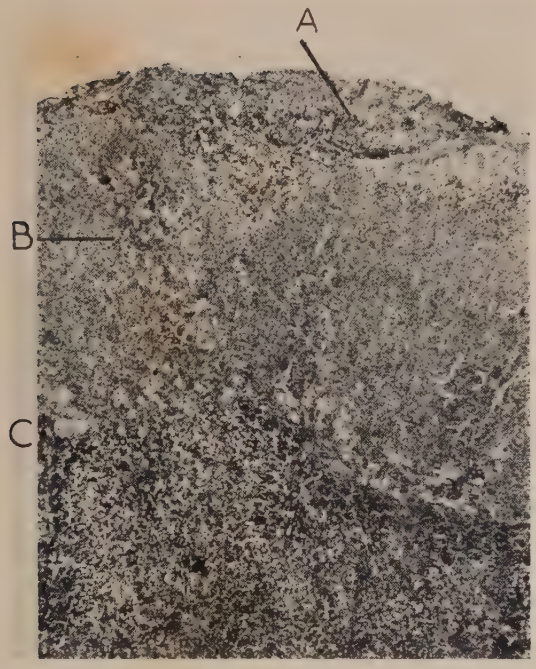
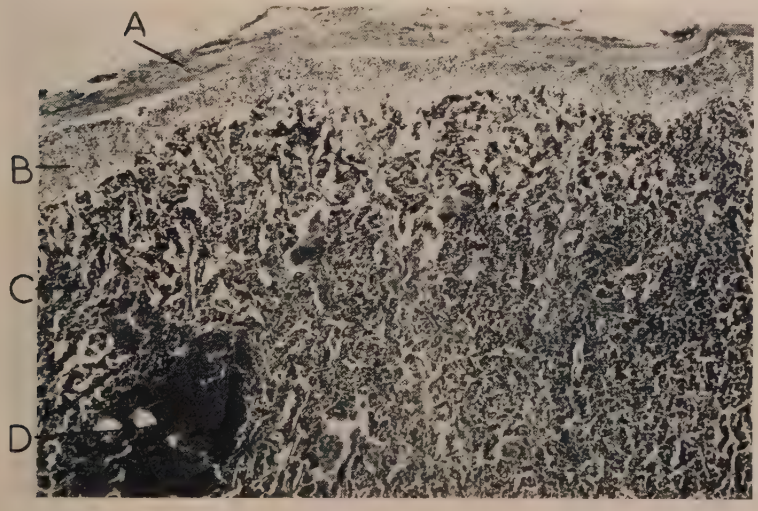
Fig. 1



Photomicrograph of the left adrenal. A nodule of hyperplasia of the cortex can be seen at (A) the zonary pattern of the cortex (B) is moderately clear and there is a distinct junction with the medulla (C). Stained H. & E. x 27.

Fig. 2



Photomicrograph of the right adrenal. Foci of disorderly cortical cells with a few central medullary cells are visible in the capsule (A). The cortex (B) is thin and shows no zonary pattern. The medulla is completely replaced by tumour tissue (C) in which there are occasional large blood spaces (D). Stained H. & E. x 27.

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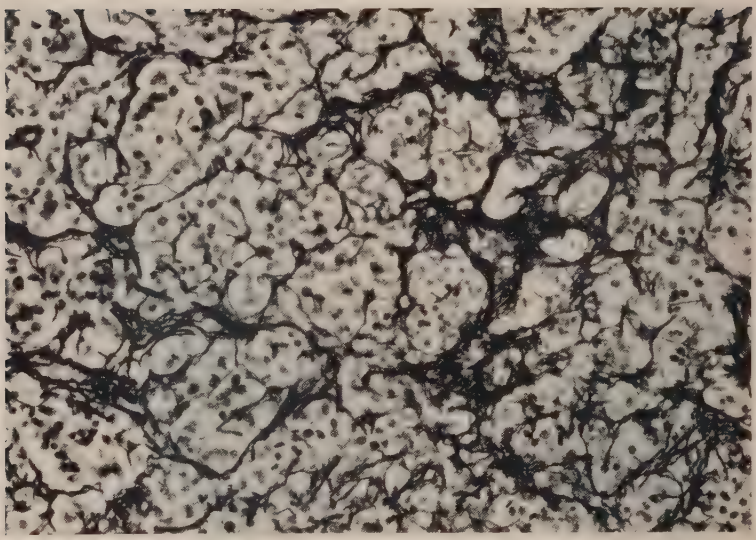
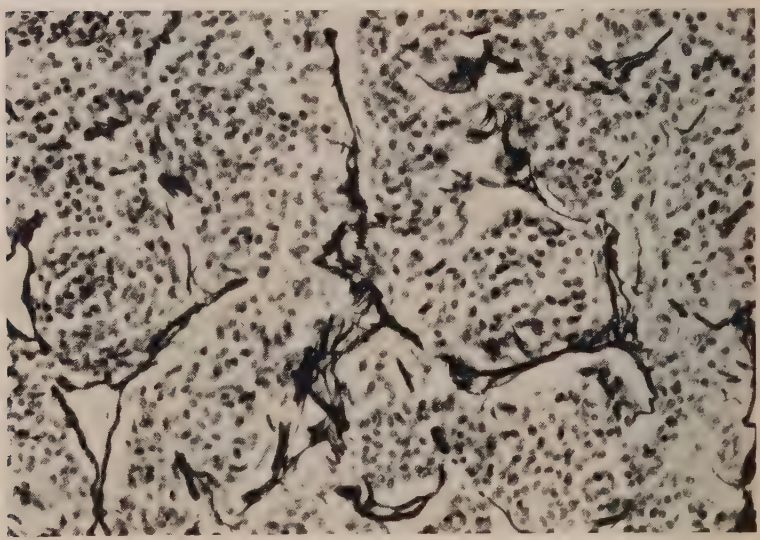
Fig. 3



Photomicrograph of the left adrenal medulla showing the reticulin network. Stained Gordon and Sweet's method. × 133.

Fig. 4



Photomicrograph of the right adrenal medulla tumour showing the reticulin network. Stained Gordon and Sweet's method. × 133.

Fig. 5

Photomicrograph of the left adrenal medulla. Schmorl's reaction. × 700.

Fig 6

Photomicrograph of the right adrenal medulla tumour. Schmorl's reaction. × 700.

Addendum

After this paper had been submitted for publication, a second case of phaeochromocytoma in a dog was brought to our attention by Dr. H. V. Coates of the Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies.

The clinical history of this 10 y.o. male Great Dane was of salivation from the left side of the mouth which became progessively worse over some weeks. The saliva drools contained blood for one week prior to the dog being brought to the clinic, when examination of the mouth revealed a melanotic tumour measuring 8.5 × 4 × 3.5 cm. at the site of the openings of the zygomatic salivary gland ducts.

Following destruction by intravenous Pentothal Sodium, an autopsy was performed immediately and the material and methods employed were as in the previous case.

Post-mortem findings were similar to those of the previous case, except for the poorly encapsulated melanotic tumour in the mouth which had not metastasised, the absence of an acquired lipomatoid mass in the sternal region, and normal gall bladder, prostate and testes. As before, the left adrenal gland showed nodular hyperplasia of the cortex. The right adrenal gland weighed 25.5 g. and consisted of a mass measuring 4 cm. anteroposteriorly × 2.5 cm. in diameter, at the posterior pole of which was a protuberance measuring 1 × 1.5 × 1 cm., whilst on the anterior medial aspect there was a 1.5 cm. long process, 1 cm. in diameter. On the cut surface the posterior protuberance and the posterior pole of the main mass were covered by a 0.1 cm. thick sheet of cortical tissue The right renal lymph node measured 1.5 cm. in diameter and like the right adrenal medulla tumour, the cut surface showed soft hyperaemic tissue.

Histological examination revealed similar changes to those of the previous case, except in the following particulars:—The projection on the anterior medial aspect of the right adrenal medulla tumour showed small tongues of tumour cells projecting into blood vessels at the periphery. Fragments of the tumour caused formalin to become brown within two days. Formalin-fixed sections treated with 3 per cent. potassium dichromate for three hours before being stained H. & E. showed a faint but distinct brown colour of the granular cytoplasm. Results with 3 per cent. potassium iodate were similar. The cells of the left adrenal medulla did not show the same differences from the tumour tissue as were noted in the previous case, suggesting that they were bordering on neoplasia.

Pharmacological assay showed that the total catechol amine content of the tumour tissue was 6.12 mg./g. fresh tissue, with noradrenaline 83 per cent of the total. Only adrenaline and noradrenaline were present.

It is interesting to note that though this tumour was malignant since it was growing invasively and had metastasised to the right renal lymph node, the cells had a more adult type of staining reaction with 3 per cent potassium dichromate and potassium iodate than in the previous case and this may be related to the higher catechol amine content. Our comments on the absence of clinical signs in the previous case are also applicable to this case.

AN EPIZOOTIC OF NAIROBI SHEEP DISEASE IN UGANDA

By

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INTRODUCTION

Montgomery (1917) published a report on a tick-borne haemorrhagic gastroenteritis of sheep and goats which has since been known as Nairobi Sheep Disease (NSD). Daubney and Hudson (1931) confirmed and amplified Montgomery's results. In Kenya, the disease appears to be endemic in the Kedong Valley, the Kikuyu Reserve and the environs of Nairobi; it has also been reported from Makindu, Voi, Burra and Maktau, 100 to 200 miles southeast of Nairobi. In Uganda, the disease has been reported, mainly on clinical grounds, as the cause of a high mortality in sheep in Mengo District, in 1931-33 at Entebbe, and in 1940, at Mityana (Uganda Protectorate, 1931-41). Infestation of the sheep by ticks was noted, and the disease was transmitted experimentally to sheep and goats by the inoculation of blood, both unfiltered and after passage through a Berkefeld N filter. The splenic enlargement described as typical of the disease in Kenya (Daubney and Hudson, 1931) was stated to be absent, as was the case also in the outbreak now to be described.

In the latter part of 1955, there was an outbreak of disease among sheep at the Animal Health Research Centre, Entebbe (AHRC). Investigations showed that the cause of the illness was unlikely to be bacterial or toxic and a programme was arranged with the East African Virus Research Institute, Entebbe (VRI) to ascertain if a virus was concerned. This paper describes the outbreak, the work which established that the cause of the disease was a strain of NSD virus and some epidemiological and physiological studies.

MATERIALS AND METHODS

Methods were, in general, standard ones, but some specific details should be mentioned.

Stock virus. Whole infective sheep serum, or a 10 per cent suspension of sheep spleen or mouse brain in non-immune sheep serum (heat inactivated at 56° C for 60 min. and either concentrated or diluted 1:2 in normal saline) were used. The stock was stored at −25° C, some frozen and some lyophilized.

Diluent. Except for the serum saline mentioned above, 0·75 per cent Armour's Fraction V bovine plasma albumin in isotonic saline buffered to pH 7·4 (BPA) was used.

Experimental animals. Native Persian fat-tailed sheep, purchased in Uganda, were employed. At the time at which the epizootic occurred, the routine practice was to allow the sheep to run in a paddock by day

and to confine them in stables at night. When the experiments began, however, precautions against arthropod attack were taken; all sheep for experimental purposes were ticked on arrival, sprayed with gamma benzene-hexachloride every 3 days and confined in the stables for at least 3 weeks before use. Swiss albino mice from the colony of the VRI were used. Embryonated eggs were of Rhode Island Red, Australorp, and White leghorn breeds.

Inoculation of experimental animals. Sheep were inoculated with 1·0 ml quantities of material either subcutaneously (SC) into the soft skin of the abdomen near the groin, or intravenously (IV) into the jugular vein. For attempted virus isolation, 2 litters of 3-day-old and 1 group of adult mice were inoculated with each specimen. The infant mice each received 0·03 ml of material in 3 doses of 0·01 ml, intracerebrally (IC), SC and intraperitoneally (IP), in that order. The IC and SC doses were given following the method of Taylor, Hurlbut, Work, Kingston and Frothingham (1955). The adult mice each received 0·03 ml IC. or 0·1 ml IP.

Titrations and neutralization tests. Ten-fold dilutions of virus were used throughout and undiluted sera for antibody content. Incubation in neutralization tests was for 2 hours at 37° C. Except where otherwise stated, all tests were carried out in adult mice inoculated I.C. End-points were calculated by the method of Reed and Muench (1938).

Immune sera. Initially sheep serum which had been found to contain neutralizing substances to the agent isolated during the Entebbe epizootic was used. Monovalent sera were prepared as soon as possible by the hyperimmunization of laboratory-reared rabbits and mice, given 3 doses of live virus IP at fortnightly intervals.

Serum analysis. Serum analysis was carried out by the method of paper electrophoresis.

Haemoglobin estimation. Haemoglobin was estimated by means of the "M.R.C." Grey Wedge Photometer by the procedures recommended by Evans Electroselium Ltd.

Chick embryos were inoculated by the standard techniques described by Beveridge and Burnet (1946).

Arthropod collections. Catches were designed to recognize the blood-sucking arthropods present in the area, and particularly to assess the amount of their attack on sheep. Teams of 3 men attended animals and caught the biting insects which alighted on the animals. The possibility that small species, such as *Culicoides* spp., might be present but be missed in such catches was investigated by making catches of resting insects on dark cloths hung on hedges or under trees and by means of lighted suction traps. Ticks were collected from the sheep themselves, the collection from each animal being kept separate, and from blankets dragged over the grass of the paddock.

RESULTS

The Entebbe Epizootic

On 28 September, 1955, 13 sheep, purchased at Mityana, Mengo District, Uganda, were quartered at the AHRC with 3 adult sheep, which had been there for periods ranging from 34 to 14 months, and 1 lamb born there 6½ months previously. Table 1

shows the deaths which occurred among these sheep; NSD virus was isolated from one of them.

Sera from the above sheep taken before the epizootic were negative. Four months later neutralization tests on the sera of 7 of the 8 sheep which survived the epizootic showed that 5 were immune to NSD, one result was equivocal the serum neutralizing 0·7 log LD_{50} of NSD virus in repeated tests, and one was negative. The single sheep which gave a negative result after the epizootic died about 6 months later (28 May, 1956) and NSD virus was isolated from it at that time. It is clear, however, that some of the sheep experienced a non-fatal attack of the disease and developed antibodies.

TABLE 1

SHEEP WHICH DIED OF NSD IN THE FIRST WAVE OF DISEASE IN ENTEBBE

No.	*Category*	*Day of death counted from arrival of sheep from Mityana (28 Sep., 1955)*
3224	New	10
3230	New	12
3218	New	14
3189*	Old	22
3220	New	22
3222	New	26
3225	New	26
3213	Old	39
3221	New	40

*A serum specimen taken on the day before this animal died, and stored at—20°C yielded an isolation of virus when inoculated into mice 3 months later.

Clinical Features and Pathology

Table 2 summarizes some of the features of the disease as seen in the present outbreak. The fever, always high, sometimes exceeded 107° F. The temperature usually reached a peak 36 to 48 hours after it began to rise, remained elevated for a further 48 to 72 hours and then suddenly dropped to a subnormal level, when the animal died. The fever was not invariably of this pattern; sometimes it was remittent for 4 or 5 days, each peak reaching a higher level than its predecessor, but in these cases also, the disease terminated abruptly in death as the temperature fell to a subnormal level (Fig. 1). At about the time of the first temperature rise, sheep appeared listless, stood apart from the flock with their heads hanging down and ate only half heartedly. Diarrhoea usually appeared on the second day of illness, rapidly increased in severity and invariably became bloody. Terminally there was usually a mucoid discharge from the nostrils and respiratory distress.

TABLE 2
THE CLINICAL PICTURE OF NSD AS SEEN IN UGANDA AND KENYA

Entebbe, Uganda	*Nairobi, Kenya*
Onset of symptoms before peak of fever.	Onset of symptoms after peak of fever.
Fall of temperature sudden, perhaps because sheep moribund.	Fall of temperature more gradual.
Death follows within few hours of temperature drop.	Death often not for days after temperature falls.
Haemorrhage into gut more marked—frequently with frank blood in stool.	Stool usually bright green.
Diarrhoea and tenesmus less marked.	Diarrhoea and tenesmus more marked.
Stomach full post mortem.	Stomach empty post mortem.
Spleen seldom enlarged to twice normal size.	Spleen often six times normal size.
Greater degree of cardiac involvement.	Lesser degree of cardiac involvement.
Pulmonary oedema with tracheal froth common.	Pulmonary oedema not seen: tracheal froth rare.
Tracheitis common.	Tracheitis not reported.
Female genital tract seldom involved and and if so only mildly.	Female genital tract always involved with gross changes.

Post-mortem Examination

Brain. The meninges were congested and the brain was rather softer than normal.

Heart. In every case the pericardium contained fluid which varied in amount from 1 to 4 ml. The surface of the myocardium showed ecchymoses, especially over the coronary fat.

Abdominal viscera. Haemorrhages occurred on the surface of almost any part of the gastro-intestinal tract; they varied from submucous petechiae to frank bleeding and were most marked in the abomasum, duodenum, caecum, colon and gall bladder. In the caecum and colon, lines of ecchymosis along the tops of oedematous longitudinal folds of mucosa produced a characteristic striped appearance. The mesenteric lymph nodes were grossly enlarged, soft and unusually pale in colour, never haemorrhagic. The liver was normal in size and had the pale, mottled and pinkish-yellow appearance seen in advanced anaemia. The spleen was usually a little enlarged and frequently showed subcapsular haemorrhages. The kidneys, adrenals, pancreas and genitalia were normal in appearance. The bladder usually contained only a small quantity of dark, concentrated urine; overt blood was never noted.

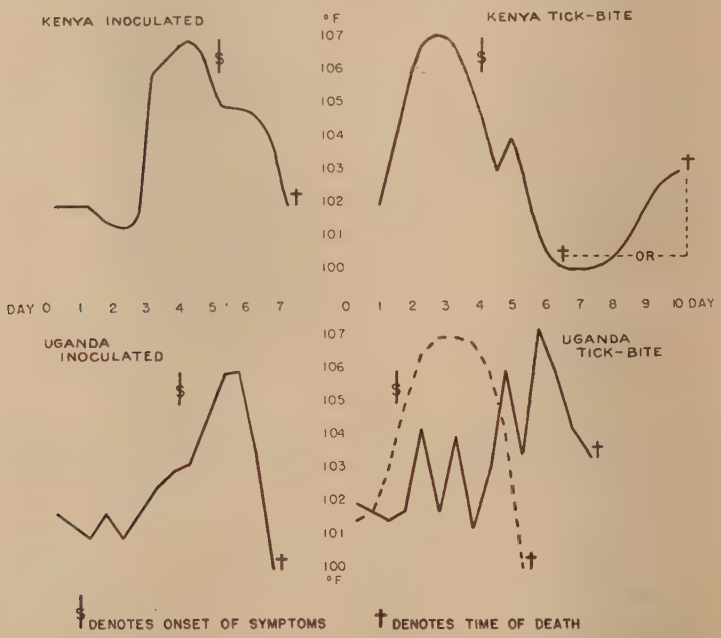
Viral and Physiological Studies

Isolations

The first isolation was made from the serum of sheep No. 3189 (Table 1). This animal exhibited the remittent type of fever and its

temperature chart has been reproduced as part of Fig. 1. A serum specimen collected on the day before the sheep died, when it had a fever of 107·2°F, and stored at −20°C for 59 days, was inoculated into 2 litters of five 3-day-old mice, and one group of adults. The adult mice remained well during a 30-day observation period but two mice were moribund in one litter (A) on the 9th post-inoculation day and two sick in the other (B) on day 10. The remaining six mice survived and were discarded on day 30.

Fig. 1



Temperature charts of sheep infected with Nairobi sheep disease. The left hand charts show the course of infections artificially produced by the inoculation of infective serum, the right hand ones those naturally induced by the bite of ticks. The upper pair are representative of the disease as seen in Kenya and are constructed from data given by Montgomery (1917) and Daubney and Hudson (1931, 1934). The lower pair represent the disease as seen in the Entebbe outbreak; the left hand chart and the broken-line chart on the right combine two actual temperature charts; the continuous line chart on the right is the actual chart of sheep No. 3189 from which the first isolation was made. Symbols indicate the times at which symptoms were most likely to appear and death to occur.

Brain material from the two moribund mice in litter A was inoculated unfiltered and after passage through a Seitz EK pad, to infant mice IC and IP and to adult mice IC; all these mice sickened on the 5th post-inoculation day and were sacrificed. Liver material

from litter A was inoculated unfiltered, IC and IP to both infant and adult mice. All were sick on the 5th or 6th post-inoculation days except the adults inoculated IP which never sickened. Brain material from the two sick mice in litter B was used successfully to passage the agent to infant and adult mice by the IC route.

To prove the origin of the agent, further inoculations of the original serum were made into infant and adult mice; eight of 19 infant mice sickened and were sacrificed on the 11th day. Of six adult mice inoculated IC, two were hyperactive on the 8th post-inoculation day, and on the 10th day, one of these two mice was found in convulsions and was sacrificed; the other recovered, and all the other mice remained well. Successful passages were made from both pooled infant brains and adult brain. Thus, it may be concluded that the agent was derived from the serum.

The picture in infant mice showed no unusual features. In adult mice, on the other hand, the clinical features were characteristic. Usually they remain well until about 3 days after inoculation when they become very active and excitable, scampering about their cages with their tails held erect or arched over the back. Soon after this phase, the fur becomes ruffled and the mice appear sick. They appear to have photophobia—many of them hide their heads—and are hyperirritable; if disturbed, they squeak loudly and jump. Mice disturbed in the latter stages of this phase go into convulsions of a characteristic type; the neck arched, the muzzle closely pressed on to the chest, the fore-limbs crossed and tightly clamped over the thorax and the hind-limbs and tail including the vertebral column hyperextended. In this posture, the mouse ceases to breathe, becomes deeply cyanosed and may die; if it does not die, deep gasping respirations follow and it slowly recovers. All the mice which die of NSD do so in the posture described above. This finding was so constant and so unlike the picture produced by any other described virus disease with the exception of lymphocytic choriomeningitis that we feel its occurrence following inoculation of unknown material from sheep or ticks constitutes grounds for making a tentative diagnosis of NSD.

In mid-April, 1956, 13 sheep arrived at the AHRC from Mityana. Neutralization tests showed that none of their sera contained antibody to NSD virus. One sheep sickened on the 6th and another on the 10th day; both died after 6 to 7 days and NSD virus was isolated from their sera. Neither of these sheep had been in the infected paddock. Nine more of these sheep succumbed to infection by NSD, after exposure in the infected paddock, between 29 and 139 days after arrival in Entebbe.

Identification of the Isolate

The Entebbe isolate was set up against antisera known to be potent against the following viruses: Pantropic Rift Valley fever

(3 strains), Lunyo (an atypical strain of Rift Valley fever), Chikungunya, Hawaiian dengue, New Guinea dengue, the Mengo strain of encephalomyocarditis, Zika, Semliki Forest, Uganda S, Bunyamwera, West Nile fever, yellow fever, Bwamba fever, Wesselsbron*, Pongola*, Simbu*, and Spondweni*. None of these antisera showed any neutralizing activity against the Entebbe isolate. Samples of immune serum to the agent were subsequently sent to the Rockefeller Foundation Virus Laboratories in New York, and tests carried out there showed it to be a member of the "B" group of Casals and Brown.

A lyophilized sample of a strain of NSD virus obtained from the Veterinary Research Laboratory, Kabete, in June, 1956, produced no clinical reaction or change of immune status when inoculated into 4 sheep, 2 of which were known to be immune and 2 non-immune to the Entebbe isolates. This NSD challenge strain was titrated in mice but proved barely viable. On passage in mice, however, it increased in activity. Tests were set up with specific NSD immune serum, stored since 1945 at the VRI, which gave the result shown in Table 3. These results indicate that the Entebbe and Nairobi viruses are strains of the same agent.

TABLE 3

Virus	*Logs virus neutralized by antisera to NSD*		
	Entebbe strain (prepared in sheep)	*Entebbe strain (prepared in rabbit)*	*Specific NSD immune serum (prepared in sheep)*
Entebbe	2·9	2·3	2·9
Nairobi	3·0	2·7	2·8

Neutralization Tests on Sera from Various Localities in Uganda

In an attempt to show whether NSD occurs in parts of Uganda other than the AHRC, a small number of sheep and goat sera were collected and tested for neutralizing substances. The distance of the localities sampled from Entebbe, and the results obtained are as follows:—Mityana; 40 miles NW; sheep 0/18. Kawanda; 30 miles N; sheep 2/10; lambs 0/2. Kamuli; 80 miles NNE; sheep 0/5; goats 1/5. Kampiringisa; 30 miles W; sheep 4/6; lambs 1/5. Mbarara; 200 miles WSW; sheep 5/13. Livestock Experimental Station, Entebbe; 1 mile S; goats 3/6; kids 1/4. AHRC; goats

*These agents have recently been isolated in the Union of South Africa. Wesselsbron was obtained from sheep (Weiss, Haig and Alexander, 1956), man and mosquitoes (Smithburn, Kokernot, Weinbren and de Meillon (1957)); Pongola (Kokernot, Smithburn, Weinbren and de Meillon (1957)), Simbu (Weinbren, Heymann, Kokernot and Paterson, 1957), and Spondweni (Kokernot, Smithburn, Muspratt and Hodgson, 1957), were all obtained from mosquitoes. The antisera to all these agents were kindly supplied by Dr. K. C. Smithburn of the Rockefeller Foundation.

4/5; kids 2/2. There was no history of illness or deaths among goats at Entebbe so the animals found immune must have undergone inapparent infections.

Sera from 8 Europeans and 2 Africans who were in contact with the animals during the Entebbe epizootic were tested. None of these contained antibodies, but the serum of one of the VRI African Staff, who had not been in contact with any known source of NSD either in the field or the laboratory, was found to contain strong neutralizing antibodies for NSD. This serum was obtained when the man was convalescent after an attack of Rift Valley fever. As 3 other RVF antisera did not show any neutralizing power for NSD, we assume that his serum was polyvalent and contained specific antibodies to NSD.

The Site of Virus in the Sheep

In order to determine the probable site of the virus in an infected animal, two non-immune young adult sheep were inoculated with NSD virus: one was sacrificed when its temperature reached 107·9°F and the other was allowed to die. A variety of specimens was obtained at both autopsies, and the results of titrations in mice are given in Table 4. The spleen and liver had high virus contents on both occasions, but, as the disease progressed, the relative level of virus fell in the blood and abomasum, while it rose in the mesenteric lymph glands, the heart and the caecum (Table 4).

TABLE 4

Organ or secretion	*Titre of virus as mouse log LD_{50}/0·03 ml*	
	Sacrificed sheep ♀	*Dead sheep* ♂
Spleen	5·2	3·8
Blood (serum)	4·6	1·2
Liver	4·6	3·4
Mesenteric lymph gland	2·6	4·5
Kidney	2·5	2·6
Inguinal lymph gland	1·8	Not tested
Urine	1·6	Not available
Heart	1·4	2·2
Abomasum	1·0	None
Uterus	0·8	—
Caecum	None	3·4
Brain	None	Not tested
Gall bladder	None	Not tested

The Susceptibility of Mice

Adult mice showed no clinical reaction to IP inoculation of NSD virus, whether the infective material was sheep serum or mouse brain. Multiple IP inocula, however, led to the production of neutralizing antibodies. Adult mice became sick and died following the IC inoculation as did infant mice when inoculated either IC or IP. The IC route in infant mice was associated with a higher

titre and a shorter incubation period than was the case with the other methods. Titrations of the brains, livers and blood of moribund adult mice showed that titres as high as 7·0 log LD_{50}/0·03 ml occurred in the brain, while the liver and blood never contained more than a trace of virus.

Attempted Cultivation in Embryonated Eggs

Inoculations of NSD virus were made into embryonated eggs of various ages, by all the standard routes of inoculation and using high titre sheep serum, sheep spleen in serum, and mouse brain in BPA as sources of virus. As a routine procedure, chick-embryo liver was taken for inoculation into mice on the 4th post-inoculation day. No virus was recovered in these mice with one exception. One 12-day embryo received 2·0 log LD_{50} of NSD virus on the chorio-allantoic membrane. On the 4th day the embryo was haemorrhagic, particularly in the skin and liver; NSD virus was recovered by inoculation of a liver suspension into mice. It was thought that the failure of the virus to survive in embryonated eggs might be due to the eggs used being derived from hens in the infected area but substitution of other eggs, to which this suspicion did not attach, made no difference to the results.

Histopathology

Apart from changes associated with the macroscopically visible haemorrhagic phenomena, the only pathological process noted in sheep was a glomerulo-tubular nephritis.

In adult mice, the pathological process appears to be confined to the brain where there is an encephalitis of viral type in which the most marked changes are seen in the hippocampus, whose cells show swelling of the nuclei with margination of the chromatin. This appears to be an early stage in a process which causes lysis of the cell. Focal cystic areas are found where this has occurred in a number of adjacent cells. In some instances, the hippocampal cells undergo a coagulative necrosis in which the cytoplasm and nucleus merge into a homogeneous eosinophilic mass in which, at certain stages, it is possible to see a few exceedingly fine basophilic particles. Elsewhere in the brain widespread degeneration of neurones and a generalized vascular dilatation are seen. Perivascular cuffing does not occur. There is very little infiltration of the affected areas by leucocytes.

In infant mice, similar changes are seen in the brain though they are much more marked and porencephaly is common. Extraneural changes in infant mice comprise skeletal myositis and myocarditis. In both these conditions the affected muscle fibres lose their striations, become swollen and hyaline and have a much greater than normal affinity for eosin; the affected fibres become wavy and then fragment. There is an increase in the number of nuclei many of which are degenerate. These processes are followed

by phagocytic infiltration. Fragmentation of fibres in cardiac muscle does not appear to be as common as it is in skeletal muscle. No other extraneural lesions were seen.

The Blood in Infected Sheep

Cells. The blood of the 2 young sheep which were inoculated with NSD virus (see above) was examined daily. The haemoglobin contents and the total white cell counts are shown in Table 5. The sheep which died had a rather less rapid onset of symptoms than the other but nevertheless both show the same remarkable fall in the white cell count. The differential count remained unchanged throughout both series of counts except that there was a terminal increase in the number of young polymorphonuclear leucocytes in the case of the sheep which died.

Serum proteins. Paper electrophoresis was carried out on pre-infection and terminal bleedings of 8 cases of NSD in sheep (Table 6). Though the patterns obtained for the pre-inoculation specimens do not tally completely with those quoted for European-type sheep in America (Deutsch and Goodloe, 1945), they are consistent and for our purposes have been accepted as the normal for the type of sheep we used. There is little change in the general distribution of the protein fractions, though there is almost invariably a fall in total protein. This may be as great as 33 per cent though an average figure would appear to be about 20 per cent.

TABLE 5

	Sheep 1 (*sacrificed*)		*Sheep* 2 (*died*)		
Day	*Hb* g.%	*Total WBC*(10^3)	*Day*	*Hb* g.%	*Total WBC*(10^3)
0	14·3	4·4	0	10·05	4·8
1	14·3	5·3	1	10·05	5·1
2	13·1	1·4	2	10·65	4·8
At sacrifice	11·25	1·1	3	10·20	1·2
			4	9·75	0·9

The Blood-sucking Arthropods in the Area and the Isolation of NSD from Ticks

Continuous catching for 48 hours on sheep as bait failed to yield any biting insects when the sheep were undergoing the normal routine of stabling at night and running free in the paddock by day. A similar catch on 2 sheep tethered in the paddock by day and by night yielded the following biting insects: *Taeniorhynchus* (*Coquillettidia*) *fuscopennatus* Theobald, 14; *Taeniorhynchus* (*Mansonioides*) *africanus* Theobald, 5; *Taeniorhynchus* (*Mansonioides*) *uniformis* Theobald, 5; *Culex* spp., 1; Total, 25.

A similar picture was seen when catches were carried out using bovine bait. Continuous catching for 48 hours on a herd of cattle

at pasture yielded only 4 mosquitoes, but when a similar catch was made using as bait a sick animal which was lying down, 54 insects were taken. The composition of these catches is given below:

	At pasture	*Lying down*
Anopheles (*Anopheles*) *coustani* Laveran		7
Taeniorhynchus fuscopennatus		1
Taeniorhynchus africanus	2	4
Taeniorhynchus uniformis	2	34
Culex (*Culex*) *poicilipes* Theobald		1
Culex (*Neoculex*) *rubinotus* Theobald		6
Tabanus par Walker		1
	4	54

TABLE 6

COMPARISON OF PROTEIN CONSTITUTIONS OF THE PRE-INOCULATION AND TERMINAL SERA OF SHEEP INFECTED WITH NSD

Specimen number	*Total protein g.%*	*Percentage of total protein*				
		Albumin	*Globulin fractions* α1	α2	β	γ
3248A	7·20	51·6	2·0	7·1	18·4	20·9
B	7·20	45·2	2·3	7·8	18·3	26·4
3246A	6·94	44·3	4·6	11·3	16·4	23·4
B	4·70	47·1	3·1	9·5	20·6	19·7
3247A	6·57	43·0	2·2	7·7	15·6	31·5
B	5·66	40·4	1·7	7·5	18·2	32·2
3251A	6·60	49·6	2·9	6·2	19·7	21·6
B	5·50	46·1	2·7	8·1	17·5	25·6
3252A	7·67	44·0	2·4	8·8	18·3	26·5
B	6·02	45·9	1·7	5·4	16·7	30·3
3241A	7·12	35·7	1·4	2·8	21·0	39·2
B	5·73	38·2	1·7	9·1	14·5	36·5
3244A	7·67	40·9	1·5	4·5	22·6	30·5
B	6·10	41·0	1·6	6·7	17·0	33·7
3245A	6·39	39·3	1·9	7·7	21·8	29·3
B	5·30	36·9	1·7	10·2	21·2	30·0

A—Pre-inoculation specimen.
B—Terminal specimen.

From these results it was judged exceedingly unlikely that the sheep disease was mosquito-transmitted and other methods of capture were introduced in an attempt to discover other kinds of biting insect, such as *Culicoides* spp., which might be concerned.

Two lighted suction traps were run for 2 nights and yielded the 41 biting insects listed below: *Hodgesia* sp., 1; *Ficalbia* (*Mimomyia*) *perplexens* Edwards, 1; *Taeniorhynchus* (*Coquillettidia*) *metallicus* Theobald, 2; *Taeniorhynchus fuscopennatus*, 19; *Taeniorhynchus africanus*, 2; *Taeniorhynchus uniformis*, 5; *Aëdes* (*Stegomyia*) *africanus* Theobald, 1; *Aëdes* (*Aëdimorphus*) *argenteopunctatus* Theobald, 1; *Culex* (*Neoculex*) *rima* Theobald group, 1; *Culex* (*Culex*) *univittatus* Theobald, 2; *Stomoxys* sp., 6; Total, 41.

A suspension made of these insects in BPA produced no signs of illness when inoculated into mice. Black cloths pinned to tree trunks and hung on hedges were inspected for resting insects at 15 minute intervals from mid-afternoon to mid-morning. This procedure failed to produce any biting insects.

It was concluded that no flying insect was likely to be the vector of the disease and attention was then turned to ticks. *Amblyomma* and *Rhipicephalus* spp. were found to be common. Adults and nymphs were found on sheep while numerous larvae and a few nymphs could be collected by blanket drags in the paddock. In May, 1956, some sheep which had been in the infected paddock for about a week were inspected for ticks. Especial interest attaches to one of them, sheep 3248, and to the ticks, 2 adult female and 7 gorged nymphal *Rhipicephalus* sp., which were collected. The animal became ill 5 days and died 10 days after the ticks were collected from it; NSD virus was isolated from its serum during its illness. The virus was isolated also from a suspension made of the ticks 12 days after their removal from the sheep. These occurrences correspond very well with the information given by Daubney and Hudson (1931) on the transmission of NSD by *Rhipicephalus appendiculatus* Neumann and indicate that the inoculated ticks were members of the group which infected the sheep.

Many ticks were collected in order to attempt isolations and also to carry out transmission experiments, but when it became definitely established that we were dealing with an outbreak of NSD, this project was abandoned because Montgomery (1917) and Daubney and Hudson (1931, 1934) had already shown that *Rhipicephalus* ticks are the usual vectors of the disease, and that *Amblyomma variegatum* (Fabricius) may also act as a vector in areas where *R. appendiculatus* does not occur.

DISCUSSION

The clinical picture of the disease as seen in Entebbe differs in some respects from that described from Kenya. Approximately a quarter of the naturally-acquired infections resulted in temperature charts of the type shown in Fig. 1 for sheep 3189. On two occasions, there was evidence that batches of sheep, brought to Entebbe from Mityana, conveyed the virus with them. As they all proved susceptible to the disease, it was thought that they must have become infested with infective ticks en route. It has not proved possible to

obtain further information on this point, but, as the sheep were transported by motor-truck, they could only have become infected from tick harbourages on the truck, or if they were unloaded at some point in the journey.

Bugyaki (1955) has published a preliminary report of a tick-transmitted filterable virus affecting sheep at Kisenyi in the Belgian Congo. Clinically, the condition would appear to be very similar to NSD and the only method of differentiating the two would be on an immunological basis. He states that the only difference between NSD and the Kisenyi virus is that goats do not contract the disease, but does not state whether the possibility of the goats suffering from a subclinical infection has been ruled out; it is clear from the present results that goats may undergo a subclinical infection. It appears likely that the disease described by Bugyaki was NSD, and thus, it probably occurs considerably further west in Africa than has been previously thought.

The report of Daubney and Hudson (1934) indicates that *Arvicanthis abyssinicus nubilans* Wroughton is an ideal natural host, in that, following infection with NSD, there is a period of viraemia but no apparent illness. As this is a very common rodent in Mengo, it may well be acting as the natural host in this area, and, if this is so, it will prove exceedingly difficult, if not impossible to eradicate, especially as Lewis (1946) showed that the virus may persist for as long as 871 days in *R. appendiculatus*.

CONCLUSIONS

The features of an outbreak of disease in sheep at Entebbe, Uganda, during the latter part of 1955, are described. The causal agent was isolated by the intracerebral inoculation of mice and proved to be a strain of Nairobi sheep disease virus.

The highest concentration of virus in sheep was found in the spleen and liver.

Infant mice were susceptible by both intracerebral and intraperitoneal routes of inoculation; adult mice only by the intracerebral route. Except on one occasion, attempts to cultivate the virus in embryonated eggs were unsuccessful.

The histopatholgical changes induced by the virus were a glomerulo-tubular nephritis in sheep, an encephalitis, most marked in the hippocampus in mice, and, a skeletal myositis and myocarditis in infant mice.

The infection in sheep was accompanied by a marked leukopenia and decrease in the total serum protein.

Studies on arthropods in the area of the epizootic indicated that flying blood-sucking insects were very unlikely to have been concerned in transmission. Ticks of the genus *Rhipicephalus* and *Amblyomma* were present and virus was recovered from *Rhipicephalus* ticks removed from a sheep which subsequently developed and succumbed to the disease.

The clinical pictures of the disease in cases in Uganda and Kenya respectively are compared.

Neutralizing antibody to Nairobi sheep disease virus has been discovered in the sera of sheep and goats in several other localities in Uganda and also in a human serum.

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NOTE

A paper describing studies on Nairobi sheep disease in Kenya (Ansell, 1957), which has been received since the completion of this manuscript, describes the propagation of the virus by intracerebral inoculation in laboratory mice and reports trials of this material for vaccine use. It may be pointed out that both these particular findings have already been recorded on the basis of the present work (Weinbren, 1956).

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SEROLOGICAL INVESTIGATION IN THE STUDY OF MYXOMA AND FIBROMA VIRUSES

II. The Gel Diffusion Precipitin Test

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INTRODUCTION

The purpose of this paper is to report further studies of the antigen/antibody reactions of myxoma and fibroma using a modified gel diffusion precipitin test (Mansi, 1957 b).

MATERIALS AND METHODS

The number of tame rabbits used in this investigation was 317.

Myxoma virus. "Cornwall" or typical type (Rowe, Mansi, and Hudson, 1956): "Nottingham" or atypical type (Hudson and Mansi, 1955): Neuromyxoma (Hurst, 1937).

Fibroma Virus. Fibroma virus as used by Rowe *et al.* (1956). Inoculations were carried out by intradermal injection.

Media. The media and technique of preparation were the same as those described by Mansi (1957 b.).

Antigen. An exudate, produced by freezing and thawing slices of oedematous skin lesions collected from infected rabbits, provided a uniform antigen for use in this test. Exudates produced by one piece of tissue after 24, 48 and 72 hours did not vary in antigenic content. Whole tissues and concentrated tissue suspensions were also used.

Antibody. Sera collected from convalescent rabbits were used undiluted and unheated, unless otherwise stated.

Complement-fixation test. This test was carried out as described by Mansi (1957 a).

RESULTS

Myxoma

Precipitin Reaction

When myxoma virus antigen was exposed to a serum collected from a convalescent rabbit, three lines of precipitation were usually produced. The line nearest to the antigen basin was recorded as A, the line nearest the serum basin S, and between the two, V. The reaction appeared about four hours after filling the basins. The A line appeared first, followed by S and then V. The length and the position of the lines were related to the concentration of the particular component. The A line was distinct as compared to the other lines; the S line was usually broader and slightly blurred; the V line was sharp and thin. In a few instances a second V line (VI) occurred. In recording a weak positive reaction the letter

indicating the line concerned was placed in brackets. When no reaction occurred, it was noted as O.

Precipitin Antigen

Properties of the antigen. The exudate from the typical type of myxoma lesion was subjected to different treatments as shown in Table I. The material, before treatment reacted with a positive serum to produce the S, V, and A precipitin lines.

The V precipitin antigen component which appeared to be the most heat labile, was totally destroyed when heated for 20 minutes at 56°C, while the A component was destroyed after 5 minutes at 64°C. The S line was relatively heat stable, and appeared as a weak positive even after boiling for 60 minutes. Acid solutions below pH4 appeared to inhibit formation of the V and A lines but not the S. On the other hand, alkaline solutions higher than pH10 inhibited all three, although the V line was most affected. The V component was not affected by 10 per cent ethyl alcohol or ether, but was completely destroyed by 20 per cent of each. It was also destroyed by 10 per cent methyl or butyl alcohol. The A component was not affected by 50 per cent methyl or ethyl alcohol, or ether, but was destroyed by 50 per cent butyl alcohol. The S component was not affected by 50 per cent ether, or by 20 per cent methyl or ethyl alcohol. It was destroyed, however, by 10 per cent butyl alcohol, 20 per cent ethyl alcohol and 30 per cent methyl alcohol. One per cent. of phenol, lysol, dettol and liquid soap respectively had no effect on the antigen, whilst one per cent formalin affected the S. and V components. The effects of other treatments are recorded in Table I. Repeated freezing at −30°C. and thawing at 37°C. did not appear to affect the antigenic quality of the exudate. Decomposed antigen material retained its original qualities.

Relationship between infectivity, complement-fixing, and precipitin properties. An attempt was made to establish the relationship between the three properties of the virus. Infected material was collected and weighed, and dilutions were then made. These were inoculated into healthy susceptible rabbits, and were also subjected to the complement-fixation and precipitin tests. A comparison was made between the exudate and the tissues and their suspensions, as well as between tissues of different textures, e.g. dry, oedematous, etc. as indicated in Table 2.

The exudate from the oedematous tissue contained almost as much precipitin and complement-fixing antigen as was present in the oedematous tissue. However the infective titre of the oedematous tissue was higher than that of the exudate. In atypical disease, material collected from the oedematous or semi-dry stage appeared to contain an amount of virus, complement-fixing and preciptin antigen approximately equal to that present in the oedematous stage of the typical disease. In the very dry stage of the atypical lesion, it was found that the precipitin antigen had disappeared,

TABLE 1

THE EFFECT OF DIFFERENT TREATMENTS ON THE ACTIVITY OF THE MYXOMA PRECIPITIN ANTIGEN

Treatment		Time	Result		
			S	*V*	*A*
Heat	37°C.	5–60 minutes	+	+	+
	56°C.	5 ,,	+	+	+
		10 ,,	+	(+)	+
		20–60 ,,	+	—	+
	64°C.	5 ,,	(+)	—	(+)
		10–60 ,,	(+)	—	—
	100°C.	5–60 ,,	(+)	—	—
pH	pH very acid	10 minutes	+	—	—
	pH 4	,, ,,	+	—	(+)
	pH 5—pH 8	,, ,,	+	+	+
	pH 9	,, ,,	+	—	+
	pH 10	,, ,,	—	—	—
	pH very alkaline	,, ,,	—	—	—
Methyl alcohol	10 per cent	10 minutes	+	—	+
	20 ,,	,, ,,	+	—	+
	30–50 ,,	,, ,,	—	—	+
Ethyl alcohol	10 per cent	10 minutes	+	+	+
	20 ,,	,, ,,	+	—	+
	30–50 ,,	,, ,,	—	—	+
Butyl alcohol	10–40 per cent	10 minutes	—	—	+
	50 ,,	,, ,,	—	—	—
Ether	10 per cent	10 minutes	+	+	+
	20–50 ,,	,, ,,	+	—	+
Formalin	1–3 per cent	10 minutes	(+)	(+)	+
	4–5 ,,	,, ,,	(+)	(+)	(+)
Phenol	1–2 per cent	10 minutes	+	+	+
	3–4 ,,	,, ,,	(+)	(+)	(+)
	5 ,,	,, ,,	—	—	—
Lysol	1 per cent	10 minutes	+	+	+
	2–3 ,,	,, ,,	+	—	+
	4–5 ,,	,, ,,	(+)	—	—
Dettol	1 per cent	10 minutes	+	+	+
	2 ,,	,, ,,	+	(+)	+
	3–5 ,,	,, ,,	+	—	+
Liquid soap	1–5 per cent	10 minutes	+	—	+
Freezing and thawing Alternate freezing and thawing at 37°C. at 15 minute intervals, 9 times			+	—	+
Decomposition 14 days without a preservative at room temperature			+	—	+

and was invariably replaced by the precipitin antibody. Such material was still capable of producing a very mild infection.

It appeared that the relationship between the infectivity, complement-fixing, and precipitin properties of the virus antigen could only be based on a qualitative and not on a quantitative basis.

TABLE 2

COMPARISON OF INFECTIVITY, COMPLEMENT-FIXING AND PRECIPITIN ACTIVITIES OF THE MYXOMA VIRUS-ANTIGEN

Material	*Infectivity titre*	*Complement-fixing titre*	*Precipitin titre*
Typical type			
Oedematous tissue	10^{-9}	1/7466	1/80
Tissue exudate	10^{-8}	1/6933	1/80
Atypical type			
Oedematous tissue 9 days	10^{-7}	1/3200	1/20
Oedematous tissue 21 days	10^{-8}	1/12800	1/80
Semi-dry tissue 28 days	10^{-6}	1/12800	1/20
Dry tissue 36 days	10^{-4}	1/3200	1/40
Dry tissue (872) 36 days	10^{-6}	1/2560	1/5
Very dry tissue 39 days	10^{-1}	1/50	0
Very dry tissue 51 days	10^{-1}	1/1600	0

Precipitin Antibodies

Properties of the precipitin antibodies. Serum antibodies were found to be still active in producing a precipitin reaction after being subjected to the following treatments:—Freeze-drying for 3 years: repeated freezing at –20°C. and thawing at 37 °C. for 20 times; addition of phenol to give a final concentration of 0.5 per cent, and storing at 4°C. for at least six months; bacterial contamination and decomposition; exposure to 64°C. for 20 minutes. Haemolysed sera produced reactions, but the diffused haemoglobin tended to make reading difficult.

Relationship between the complement-fixing and precipitin properties of the antibodies. As a relationship had been demonstrated between the complement-fixing and precipitin properties of the antigen, the antibody properties were also studied.

A hundred sera, collected from rabbits at different stages of recovery and convalescence from myxomatosis, were subjected to a comparative test. They were grouped according to their complement-fixing titres, and the results of the examination were recorded in Table 3. Among the thirty-one rabbits which had lost their complement-fixing antibodies, only four had also lost their precipitin antibodies. All the sixty-nine rabbits which showed complement-fixing antibodies of different titres also contained precipitin antibody of varying quality.

It appeared that the relationship between the complement-fixing and precipitin antibodies could only be based on qualitative grounds. Further evidence is demonstrated in Table 4.

TABLE 3

COMPARISONS OF THE COMPLEMENT-FIXING ANTIBODY TITRE OF SERA AND THE ANTIBODY COMPONENTS FOUND BY THE PRECIPITIN TEST

Numbers of myxoma-immune rabbits examined	*C.F.T. titre*	*Precipitin antibody components in undiluted sera*				
		O	*(A)*	*A*	*SA*	*SVA*
31	0	4	19	8		
4	4			4		
5	8		3	2		
8	16		2	5	1	
9	32			8		1
7	64			5	1	1
7	128			4	3	
11	256			7	2	2
10	512			3	5	2
2	1024				1	1
3	2048				3	
2	4096				2	
1	8192					1

TABLE 4

COMPARISONS OF THE COMPLEMENT-FIXING ANTIBODY TITRE OF SERA AND THE ANTIBODY COMPONENTS FOUND BY THE PRECIPITIN TEST

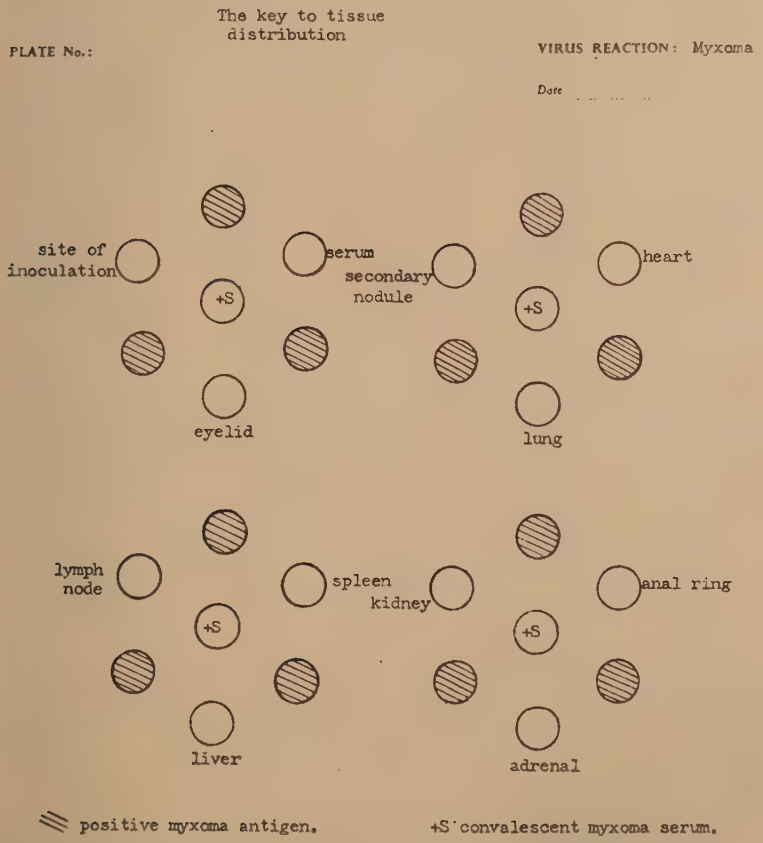
C.F.T. titre	*Precipitin reaction of undiluted sera*	*Highest dilution showing precipitin reaction*
128	SA	8
256	SA	16
256	A	32
256	A	8
512	A	16
512	A	16
1024	SA	32
2048	SA	32
2048	SA	32
2048	SA	32
4096	SA	32
8192	SVA	32

Distribution of the precipitin antibody/antigen in infected rabbits. Sera and tissues were distributed in the precipitin plates as indicated in Plate I. Convalescent rabbit serum was placed in the centre basin of each of the four units, and antigen in alternate peripheral basins 1, 3 and 5 of all units. The tissues or the sera were placed in basins 2, 4 and 6. This enabled the detection of antigen or antibody in

each tissue under test. Various tissues, as indicated in Plate I were collected from each of 139 rabbits which had been bled out. The pieces of tissue were washed individually in phenol saline before being placed in the precipitin basins. This procedure ensured that there was no residual serum antibody or antigen present on the tissue. Plates II to XIII illustrate the clinical condition of the rabbit, and the precipitin reactions produced by its serum and tissues.

Plate I

PRECIPITIN REACTION



Small pieces of tissues were cut from the carcase and placed in the appropriate basins as illustrated.

Normal. Tissues from eleven normal rabbits showed no reaction with convalescent serum. Plate II indicates the characteristic

triangular pattern of the precipitin lines which were produced by the control antigen and antibody.

Typical myxoma. Sixty-one rabbits were inoculated with the typical type of myxoma. On the third day, the precipitin antigen was demonstrable only at the site of inoculation (Plate III). By the fifth day the precipitin antigen was present in the regional lymph node as well as at the site of inoculation (Plate IV). From the seventh day onwards the antigen was found in the serum and in the majority of the tissues examined (Plate V), thus producing the typical hexagonal pattern characteristic of this condition.

Atypical myxoma. The inoculation of this type of virus produced a local reaction on the third day (Stage I) from which the precipitin antigen was demonstrable. The pattern produced was indistinguishable from that of the tissues of Stage I of the typical type of infection.

Whilst the distribution of the antigen was unchanged on the fifth day, at this time antibody (A) was detectable in the serum (Plate VI). On the seventh day (Stage II) the antigen was demonstrable in the site of inoculation in the eyelid, the secondary nodule and the anal ring, and, in a few instances, in small quantities in the lymph nodes and spleen, (Plate VII). Antibody A of the serum appeared much stronger. In Stage III, the antigen was similarly distributed as in Stage II. Antigen was never found in the lung, as was the case with the typical type. However, antibody (A) was demonstrated in the lung at this stage (Plate VIII). In Stage IV, the antigen, while present in the site of inoculation and secondary skin lesions, had disappeared from the lymph node and the spleen. Antibodies S and A were present usually in the serum, together with A in the lung, and A in the lymph node and spleen. In Stage V, the antibody level of the serum and tissues increased. Antigen, although still present in the secondary nodules had usually disappeared from the healing primary lesion (Plate IX).

The disappearance of antigen and the increase of antibody continued until ultimately antibody was demonstrable in all tissues and antigen in none (Plate X). After this stage of complete recovery, a gradual loss of antibodies from the tissues occurred, although those of the serum remained at a very high level. This stage varied considerably in duration in different animals and was called Stage VII. The loss of antibodies from the tissue continued progressively until they could be detected only in the serum. At this time (Stage VIII) the rabbit was clinically healthy and considered to be immune (Plate XII).

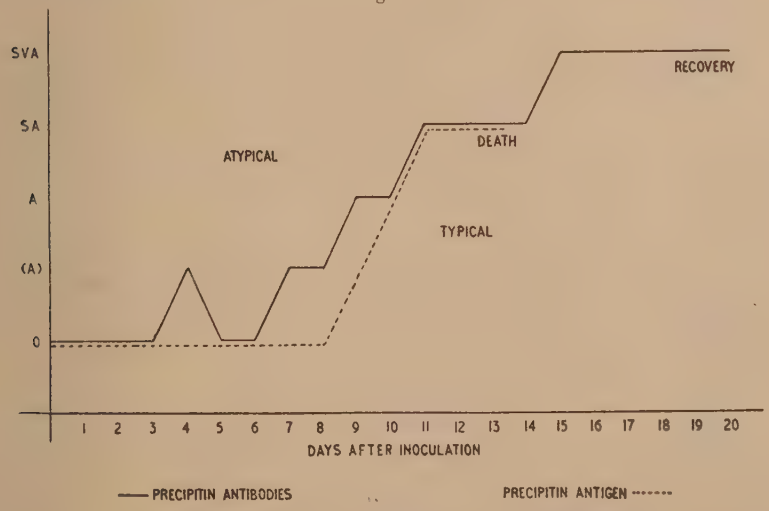
Neuromyxoma. When a rabbit was inoculated with neuromyxoma, it was found that the pattern of the precipitin reaction showed a close correlation with the atpyical type. The stages in recovery appeared to be very similar. Plate XIII shows a pattern of precipitin reaction in a neuromyxoma rabbit identical to that seen in Stage VI of an atypical infection. Table 5 presents a suggestion for the

classification of the type and stage of the infection with myxoma virus by means of the precipitin test. The times mentioned are approximate as duration of the atypical infection varied considerably from animal to animal within the same state.

Appearance of the precipitin antibody and antigen in the blood of infected rabbits. A hundred and twenty two rabbits were examined, of which 21 were bled daily. The typical type of myxoma was inoculated into 61, and the atypical into 61 rabbits.

It was found that the precipitin antigen appeared in the blood of rabbits infected with the typical type of myxoma at the beginning of the second week and usually persisted until death. None of the 61 rabbits inoculated with the atypical type showed any antigen in their blood. In these cases, the precipitin antibody was first observed by the end of the first week. The first precipitin line to appear was the A line. The reaction increased subsequently to two lines S and A, and finally to S, V and A. The S line never occurred by itself (Fig. 1).

Fig. 1



The appearance of precipitin antibody/antigen in the blood of myxoma infected rabbits.

Persistance of the precipitin antibodies in immune rabbits. Among the 178 rabbits which were examined for the presence of precipitin antibodies in their sera over a period varying from 1 to 34 months after infection, it was found that the S, V and A lines were usually present from 1 to 4 months, the S and A from 1 to 9 months while the A line persisted up to 23 months. It was possible to demonstrate the (A) line up to 34 months after infection (Table 6).

TABLE 5

SUMMARY OF THE RESULTS OBTAINED FROM TESTING SERA AND TISSUES OF HEALTHY, TYPICAL AND ATYPICAL MYXOMA INFECTED RABBITS

Clinical condition	*Nos. of rabbits examined*	*Presence of precipitin*									
		Antigen in					*Antibody in*				
		blood	*L.L.*	*spl/ln*	*lung*	*sec.L*	*blood*	*L.L.*	*spl/ln*	*lung*	*sec.L*
I. Healthy (susceptible)	12	—	—	—	—	—	—	—	—	—	—
		0	0	0	0	0	0	0	0	0	0
II. Typical infection	61										
1) 1st stage early 3-5 days	3	—	+	—	—	—	—	-	-	—	—
		0	3	0	0	0	0	0	0	0	0
2) 2nd stage beginning of generalisation 6-8 days	5	+	+	+	—	±	—	—	—	—	—
		3	5	4	0	1	0	0	0	0	0
3) 3rd stage late infection ending with death 9-15 days	53	+	+	+	+	+	—	—	—	—	—
		48	53	53	47	53	0	0	0	0	0
III. Atypical	61										
1) 1st stage early 3-5 days	4	—	+	—	—	—	±	—	—	—	—
		0	4	0	0	0	2	0	0	0	0
2) 2nd stage beginning of generalisation 6-8 days	8	—	+	+	—	+	+	—	—	—	—
		0	8	6	0	8	6	0	0	0	0
3) 3rd stage early recovery 9-20 days	6	—	+	±	—	+	+	—	—	+	—
		0	6	3	0	6	6	0	0	5	0
4) 4th stage 21-30 days	4	—	+	-	—	+	+	—	+	+	—
		0	4	0	0	4	4	0	3	4	0
5) 5th stage 31-40 days	6	—	±	—	—	+	+	+	+	+	—
		0	2	0	0	6	6	4	4	6	0
6) 6th stage 41-50 days late recovery	8	—	—	—	—	±	+	+	+	+	+
		0	0	0	0	2	8	8	8	8	8
7) 7th stage after 50 days	13	—	—	—	—	—	+	+	±	+	+
		0	0	0	0	0	13	7	3	7	6
8) 8th stage Immune. Clinically healthy	12	—	—	—	—	—	+	—	—	—	—
		0	0	0	0	0	12	0	0	0	0

KEY TO TABLE 5

+ = antigen/antibody present in over 50 per cent of examined rabbits.
— = no antigen/antibody present.
± = antigen/antibody present in 50 per cent or less of examined rabbits.
L.L.: site of inoculation, *spl.:* spleen, *ln:* lymph node (regional).
Sec.L.: secondary lesion (nodule).

TABLE 6

THE PERSISTENCE OF PRECIPITIN ANTIBODIES IN THE SERA OF MYXOMA IMMUNE RABBITS

Month after infection	*Number of rabbits examined*	*Precipitin antibody components present in examined sera*					
		SVA	*SA*	*S*	*A*	*(A)*	*O*
1	15	7	3		5		
2	20	4	6		4	2	4
3	11	3	3		3	2	1
4	18	1	8		4		5
5	5		1		2		2
6	9				7		2
7	7		1		6		
8	12	1	1		9	1	
9	20		1		13	2	4
10	5				3	2	
11	14				7		7
12	8				7	1	
13	7				5	2	
14	2				2		
15	2					1	1
20	3				3		
21	2				1		1
22	4				3	1	
23	3				2	1	
25	3					3	
26	1					1	
27	2					2	
28	1					1	
32	1				1		
33	1						1
34	2					2	

Fibroma

Tissues were collected from five fibroma infected rabbits and tested similarly to that described for the myxoma infected rabbits A characteristic precipitin pattern was produced. Neither antigen nor antibody occurred in any of the tissues examined, with the exception of the site of inoculation, where fibroma antigen was found, and the serum, where fibroma antibodies were seen to be produced.

DISCUSSION

The gel diffusion test provides a simple method for the examination of both sera and tissues for the presence of myxoma antibody and antigen. The specificity of the reaction was shown by the fact

that precipitin lines were only produced when myxoma antiserum was exposed to myxoma antigen. Normal serum placed opposite myxoma antigen never produced these lines. By the alternate arrangement of the material under test and the control antigen, it was possible to examine for the presence of both antibody and antigen. It appeared that the behaviour of the three components (S V, and A) of the myxoma precipitin antigen, varied under different treatments.

The results of comparative tests showed a qualitative relationship between the complement-fixing and precipitin antigens, and between the corresponding antibodies. A consistent pattern was also obtained when the tissues of rabbits, at various stages of disease, were examined by the precipitin test. The appearance and distribution of the antigen/antibody in the tissues were different in typical and atypical types of infection.

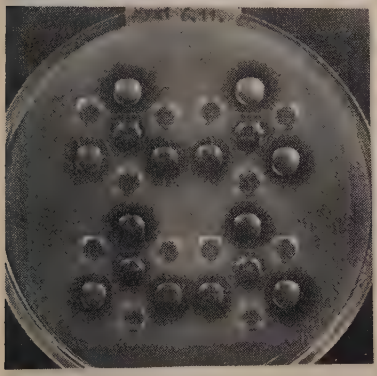
After inoculation of the typical type of myxoma virus it appeared that the antigen was produced locally at the site of inoculation, and was later demonstrable in the regional lymph node. This was followed, as generalisation of the disease occurred, by the appearance of the antigen in the serum and the spleen, and later in all the internal organs and the secondary skin lesions of the body. After the appearance of antigen in the serum and all the tissues, death followed in 2 to 5 days.

In atypical myxoma, the antigen appeared at the site of inoculation, then in the regional lymph node and later when generalisation occurred, in the internal organs, but never to the same extent as encountered in the typical form. The precipitin antigen was present apparently in large amount in the secondary lesions, but never in the serum or the lungs. Precipitin antibody appeared in the serum early in the infection (3 to 4 days after inoculation) and then more strongly after 6 to 7 days. Antibodies were demonstrated in the tissues, usually first in the lung and then in the heart, kidney, spleen, and lymph nodes. As the lesion at the site of inoculation and the secondary skin lesions dried and formed scabs, a change in the precipitin reaction pattern occurred, characterised by the gradual appearance of the precipitin antibodies in the skin lesions. With complete recovery, the antibody disappeared from the internal organs and skin lesions, but still remained at a high level in the serum.

No correlation seen could be detected between the number of antibody lines present in the blood and tissues, nor between the individual tissues, which argues against the possibility that the circulating blood plasma is the common factor producing this reaction.

Rabbits infected with the neuromyxoma strain showed precipitin reaction patterns very similar to those of the atypical type of myxoma.

A study was made of the appearance of the precipitin antibody



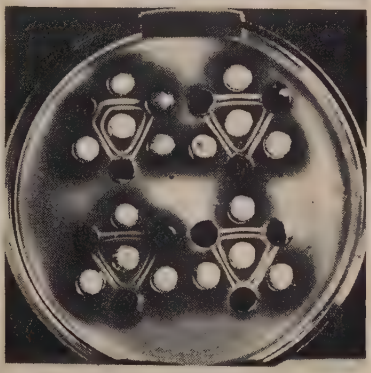


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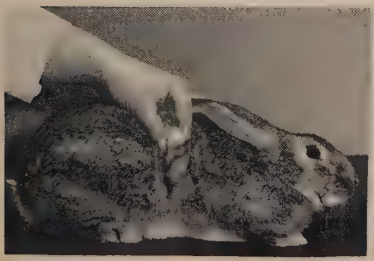
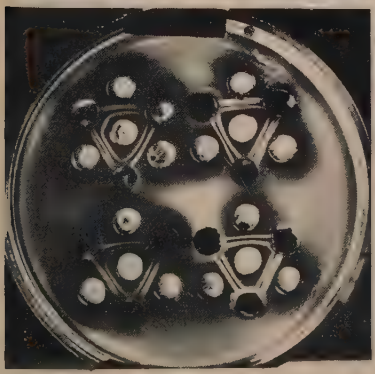
PLATE 3.





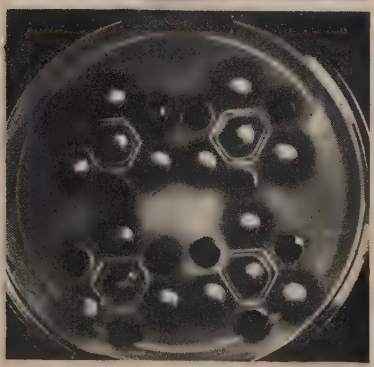


PLATE 4.

PLATE 5.

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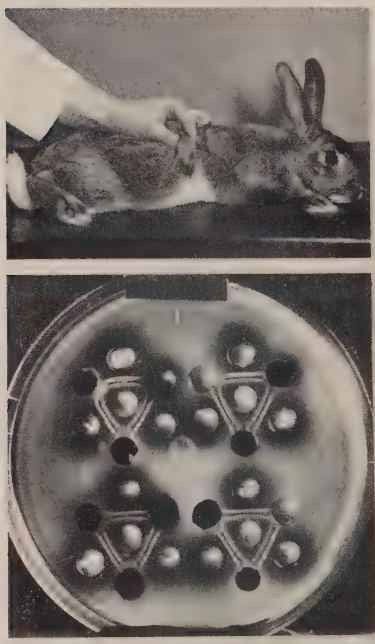

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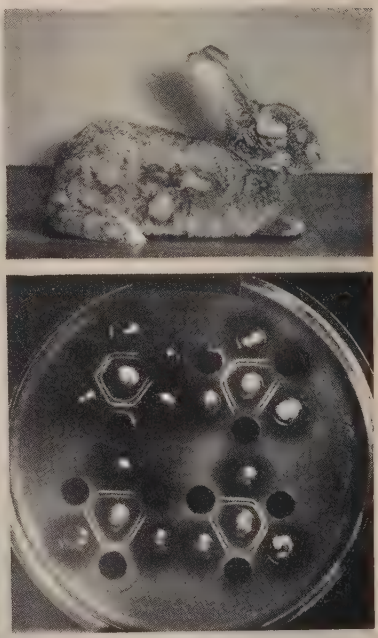

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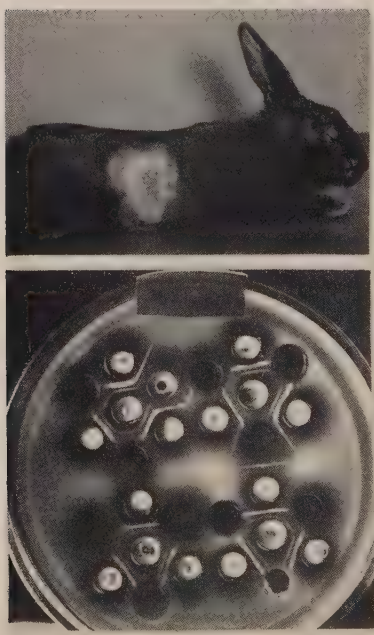

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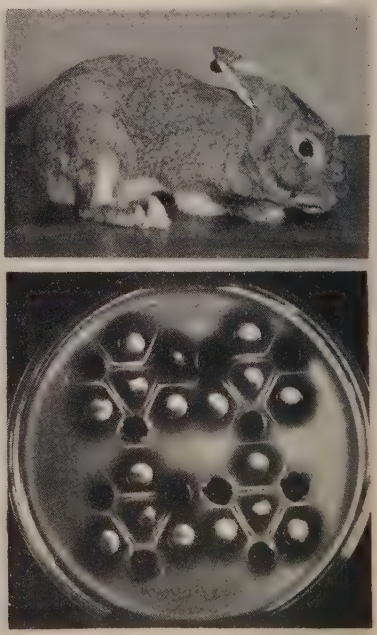

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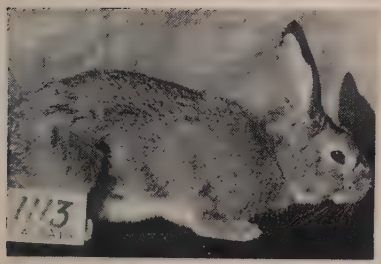

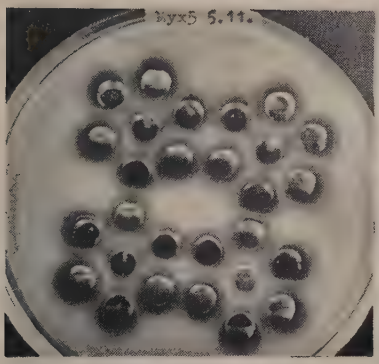

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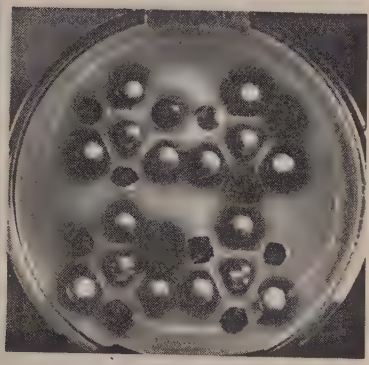

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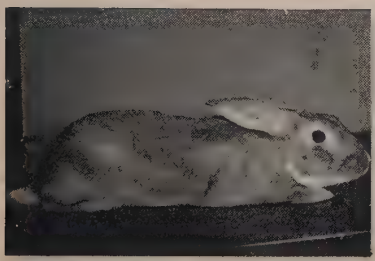

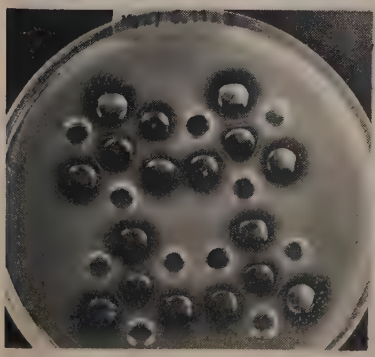

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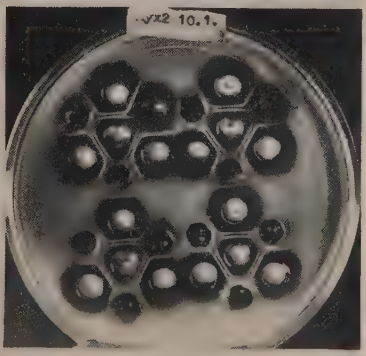

PLATE 13.

PLATE 14.

and its persistence in myxoma infected rabbits and the results showed a variation according to the severity and stage of infection. Precipitin antibody was demonstrable 34 months after primary infection.

Materials collected at an early stage from the oedematous lesions of rabbits infected with typical and atypical myxoma, and neuromyxoma, were all found to contain the S V, and A components of the myxoma precipitin antigen. It can therefore be concluded that an identical antigen is present, at this early stage of infection, in all three types of myxoma.

The relationship between myxoma and fibroma has been discussed in a previous paper (Mansi, 1957 b.), in which it was concluded that the precipitin antigens of myxoma and fibroma were related but not identical. It appears that the fibroma precipitin antigen is only produced at the site of inoculation which supports previous observation on the distribution of the fibroma complement-fixing antigen in infected rabbits.

CONCLUSIONS

This paper describes the result of the application of the gel diffusion precipitin test to the study of the myxoma virus antigen-antibody reaction. It has been possible by this technique to examine sera or tissues for the presence of either antigen or antibody or both at the same time. Some of the properties of the precipitin antigen and antibody are reported.

A study of the appearance and persistance of the precipitin antibody in myxoma infected rabbits was made.

A suggestion is made for the identification of the type and stage of infection in myxoma infected rabbits based on characteristic precipitin patterns produced by serum and tissues.

The antigenic identity of the different types of myxoma is reported.

ACKNOWLEDGEMENT

I am grateful to Mr. E. E. Clements for the photographs.

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KEY TO PLATES

Plate 2. A normal rabbit, susceptible to myxomatosis.

Plate 3. Early typical myxomatosis infection.
Stage I—precipitin antigen in site of inoculation only.

Plate 4. Early typical myxomatosis infection.
Stage II—precipitin antigen in site of inoculation and local lymph node.

Plate 5. Late typical myxomatosis infection.
Stage III—precipitin antigen in all skin lesions, serum and internal organs.

Plate 6. Early atypical myxomatosis infection.
Stage I—precipitin antigen at site of inoculation, antibody in serum.

Plate 7. Atypical myxomatosis infection.
Stage II—preciptin antigen at site of inoculation, in secondary nodules, lymph node and spleen, antibody present in the serum only.

Plate 8. Atypical myxomatosis infection.
Stage IV—precipitin antigen at site of inoculation, and in secondary nodules. Antibody in serum, lungs, and beginning to appear in other organs.

Plate 9. Atypical myxomatosis infection.
Stage V—precipitin antigen only present in secondary nodules; antibody in serum, site of inoculation, some secondary nodules and all internal organs.

Plate 10. Atypical myxomatosis infection.
Stage VI—precipitin antibodies in all tissues tested; antigen in none.

Plate 11. Atypical myxomatosis infection.
Stage VII—antibody beginning to disappear from some tissues.

Plate 12. Atypical myxomatosis infection.
Stage VIII—an immune rabbit, antibody present in serum only.

Plate 13. Neuromyxoma infected rabbit.
Late recovery, antibody present in all skin lesions, serum and in all the internal organs tested.

Plate 14. Fibroma infected rabbit.
Antibody in the serum; antigen present at site of inoculation only. In this plate the controls were fibroma antigen and immune serum. A fainter reaction was produced than that seen in those plates where myxoma controls were used.

BRACKEN POISONING IN CATTLE

By

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INTRODUCTION

A disease of cattle associated with eating bracken (*P. aquilinae*) has been recognized for many years. The literature has been reviewed by, among others, Evans, Evans, and Hughes (1954). Nowadays, the marked similarity between bracken poisoning and radiation sickness can be noted.

Stockman (1917) mentions blood trickling from the eyes as being common; we have never seen this. Evans, Evans and Hughes (1954) describe the clinical picture of bracken poisoning in cattle. Incoordination has not been present in their cases but, in North West England, swaying in the stationary animal and a peculiar swinging motion to the walk are often marked symptoms. Schofield (1947) reported leucopenia and agranulocytosis in natural cases. Faulds, quoted by Robinson (1947), considered the condition is a toxaemia and that the cause of death is thrombocytopenic purpura. Perkins (1950) in experiments commenced in 1928, and Evans, Evans and Hughes (1954) found that steaming bracken for one hour makes it harmless to cattle. Naftalin and Cushnie (1956) were unable to confirm this. This variation in experimental results may be due to different properties of different samples of bracken. Raafat (1950) showed that bracken ash is not toxic and that the HCN content of bracken probably plays no part in producing bracken poisoning. Thomas, Watkins, Evans and Evans (1955) showed that thiaminase is contained in the protein fraction of bracken and this fraction does not affect the bone marrow. The toxin which causes marrow aplasia is in the non-dialysable, non-protein fraction.

Search of the literature shows curiously little investigation of the clotting defect in bracken poisoning. Moon and Raafat (1951) found single stage prothrombin time to be normal even when plasma was diluted 1:8. Evans, Evans and Hughes (1954) reported normal values for clotting time and plasma fibrinogen and increased bleeding times during the terminal stages. Practitioners who have given injections to field cases cannot have failed to notice that blood runs from the site of injection for many hours.

Work described in this paper was designed to discover if thrombocytopenia alone is responsible for the haemorrhagic syndrome.

MATERIALS AND METHODS

Four yearling heifers (1-4) which were believed never to have eaten bracken were purchased. During a six week period normal blood values were established for the animals concerned. Thereafter all were housed separately, bedded on peat moss and fed up to 4 lb. of a proprietory concentrate daily. In addition 1 received hay, 2 was given a mixture of equal parts hay and bracken, 3 had a mixture of 30 per cent

hay and 70 per cent bracken, while 4 received a mixture of 60 per cent hay and 40 per cent bracken. In every case the fodder was given *ad lib.* All food given was weighed and residues were also recorded. The bracken used in this trial was sun-dried and baled on a hill pasture in the Pennines in October 1956. It was used in January and February 1957. None of the animals liked eating it.

Temperature, pulse and respiration were recorded twice daily on each heifer and a record was kept of any symptoms which appeared.

Glassware was all treated with silicone and blood was withdrawn into 3·8 per cent trisodium citrate three times weekly (more frequently when disease had developed). It was subjected to the following examinations.

Haemoglobin, R.B.C's and W.B.C's were estimated by conventional methods; platelets were counted by the method described by Maspes and Jamra (1955).

Wintrobe's haematocrit tubes were spun at 3,000 r.p.m. for one hour to get the packed cell volume and sedimentation rate was measured by Wintrobe's (1951) method.

A spring loaded lancet was set at 1 cm. to find the bleeding time.

Prothrombin consumption test was carried out by the method of Whitby and Britton (1953). The technique for single stage prothrombin time was essentially as described by Quick (1942), though the modification of Biggs (1951) was used.

Fibrinogen for the thrombin generation test was Armour laboratories' bovine fraction 1 and the technique used was that of Macfarlane and Biggs (1953).

Thromboplastin generation was measured by the method described by Biggs and Douglas (1953), but plasma was diluted 1/50 because there appears to be an inhibitor in bovine plasma.

Fibrinogen and total plasma proteins were estimated by the biuret method, using a Lovibond comparator.

RESULTS

Clinical Symptoms

All four animals developed ringworm during the course of the experiment.

Heifer 1 (Control) remained in good health throughout the trial.

Heifer 2 (equal parts hay and bracken) ate 88¾ lb. bracken during 36 days, after which bracken was withdrawn. Clinical bracken poisoning developed two days later when the temperature rose to 107°F. and remained above 103°F. for six days. Terramycin (500 mg.) was given intravenously as soon as pyrexia appeared. Four days after the onset of symptoms, she was bleeding heavily from the nostrils and refusing all food. Scrotonin (5-hydroxy-tryptamine) and ethanolamine phosphatide had been considered as possible methods of controlling the bleeding but sufficient supplies were not available. Egg lecithin contains some ethanolamine phosphatide and 0·8 g. in 50 ml. saline was given as an intravenous injection when bleeding was profuse; it immediately produced very severe dyspnoea, some swaying and profuse lachrymation. These effects lasted for about ten minutes, after which the animal reverted

to its former apathetic state. Haemorrhage was not affected. On the following morning it was moribund. A transfusion of 2 litres of blood from the control heifer controlled the bleeding within 4 hours. She refused food for a further day (making 4 days in all) after which she made a fairly rapid recovery.

Heifer 3 (30 per cent hay, 70 per cent bracken) ate 85¾ lb. of bracken in 36 days after which bracken was withdrawn. On the 37th day, her temperature was 104°F. and eventually rose to 106°F; it remained above 103°F. for 9 days. Terramycin (500 mg. intravenously) was given on the 37th day. Blood was present in the nostrils for 3 days during the period of pyrexia, but the bleeding never became serious. She became depressed and listless, but never refused all food. Her recovery was very rapid after the temperature dropped.

Heifer 4 (60 per cent hay, 40 per cent bracken) ate 47¾ lb. bracken during the first 18 days. Her platelet count during this period had fallen from 6 x 10^5 to 1·27 x 10^5 and she was very depressed. Bracken was withdrawn from the diet and she gradually recovered, though her platelet count had fallen to 0·31 x 10^5 three days after withdrawal of the bracken.

Haematology

Haemoglobin estimations, R.B.C. counts, W.B.C. counts and platelet counts were undertaken merely to confirm that we were, in fact, dealing with bracken poisoning. Our results with field cases have always been similar to those obtained by, among others, Evans, Evans and Hughes (1954) and Naftalin and Cushnie (1956), with the exception that leucopenia in field cases appears to be of rather later onset than in the published experimental work. Certainly from the point of forecasting probable onset of the disease platelet counts appear to be of the greatest value. Platelet counts in this trial are shown in Fig. 1, from which it will be seen that onset of the clinical disease in 2 and 3 could have been forecast some two weeks and one week respectively before symptoms actually appeared. W.B.C. counts dropped slightly a few days before onset of symptoms.

Packed cell volumes were not very informative. As might have been expected, they fell by some 15 per cent (from about 35 per cent to 20 per cent) during the course of the clinical disease, but recovery to normal was rapid.

Bleeding time rose from the normal of not more than six minutes to considerably more than 30 minutes 2 days before symptoms became well established. In some cases, bleeding from the puncture would stop in less than 10 minutes, but was so profuse that huge drops of blood rolled down the flank during the bleeding time.

Prothrombin consumption was a very unsatisfactory test in our hands. Frequently the control animal gave an index of more than 100 and the range in the control was from 4 to 323. It appears probable that with bovine blood the serum should be incubated for

a period considerably in excess of 50 minutes. Macfarlane (1956) considers that thrombin generation is more informative than prothrombin consumption, so the test was abandoned. Single stage prothrombin time was always normal.

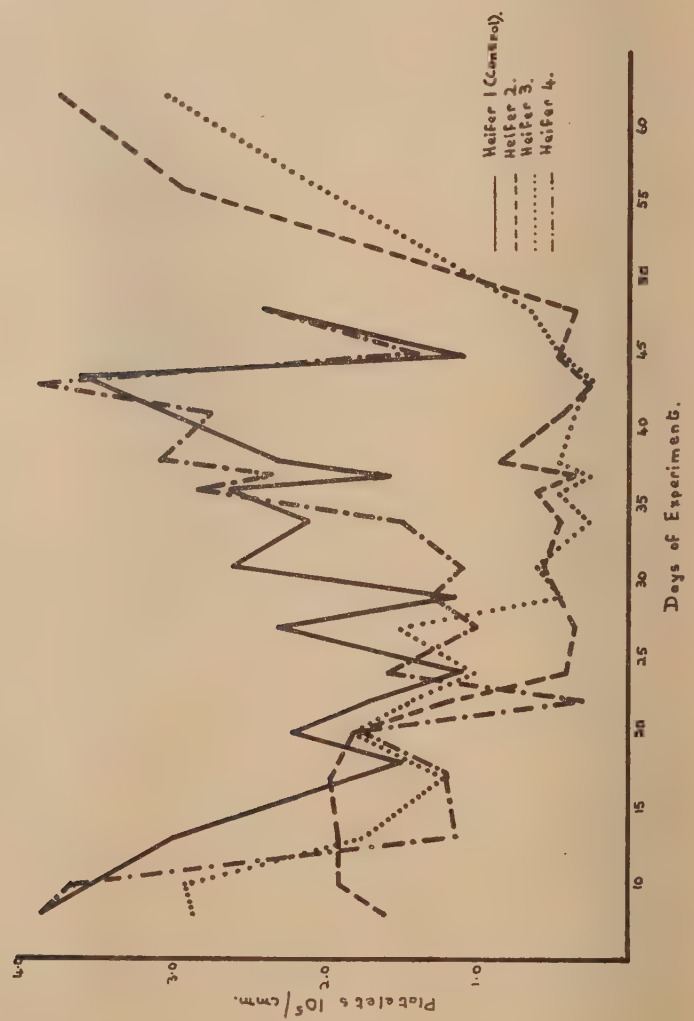
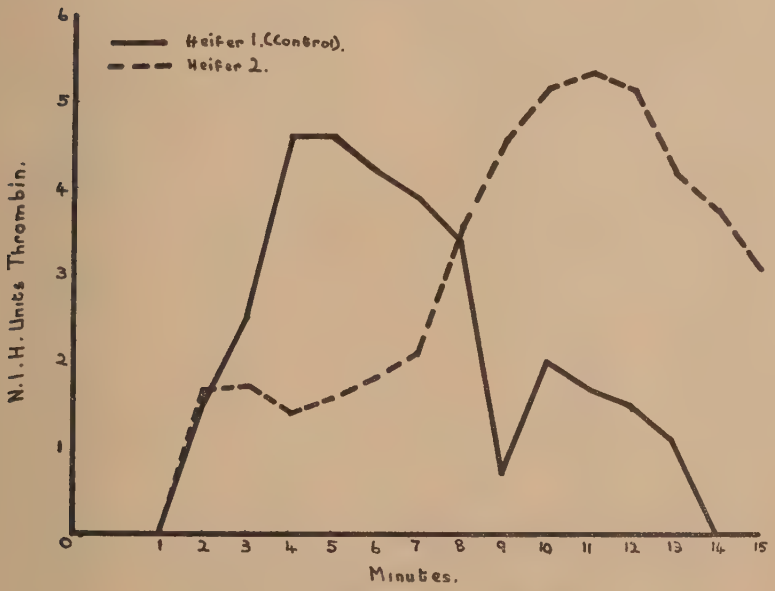


FIGURE I.

Thrombin generation gave results which were difficult to interpret. Fig. 2 shows the thrombin produced by 2 during the height of the disease, together with thrombin produced by the control. The areas enclosed by the graph show that, during the course of a disease marked by a gross clotting defect, more thrombin

than normal was produced, although production was markedly delayed. It would be cumbersome to report thrombin generation in detail, but this graph has been chosen as giving a fair picture of the difference between normal and affected animals. The clot obtained in thrombin generation tests from 2 and 3 differed markedly from that of the two animals which were not receiving bracken. Instead of the normal floppy blob which was rather troublesome to handle, the clot was swollen, rounded and shiny—not unlike a cherry.

FIGURE 2.



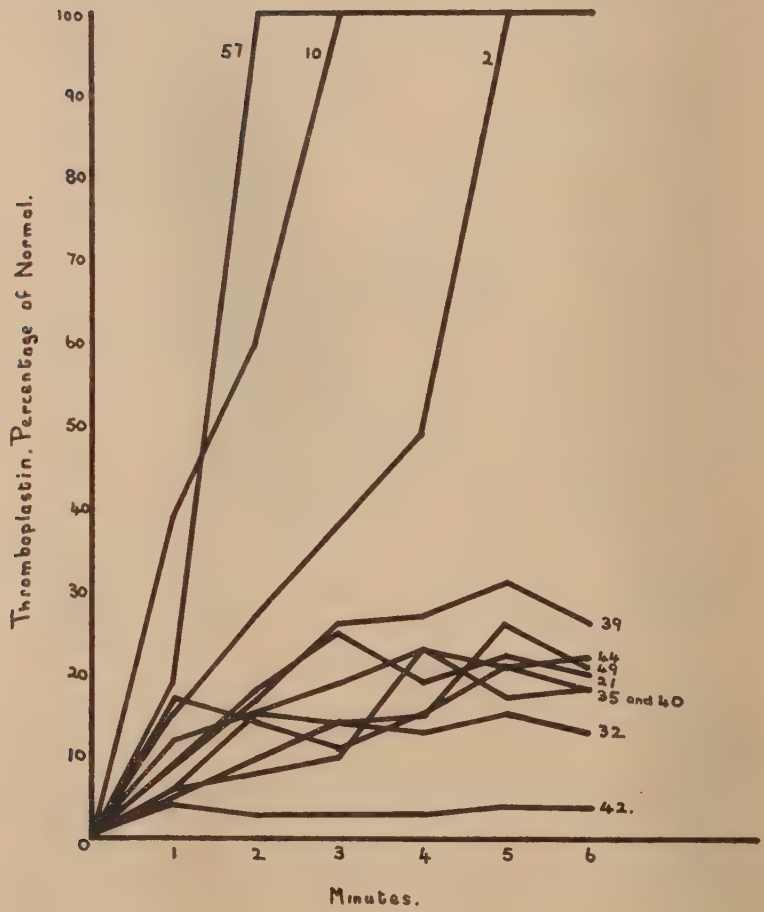
Figs. 3 and 4 show the progressive deterioration of thromboplastin generation in 2 and 3 as the effect of eating bracken became apparent.

In Fig. 5 an attempt has been made to correlate thromboplastin generation with platelet counts. The thromboplastin curve has been made by taking the arithmetic mean of the six percentages of each test. Fig. 6 shows the result of replacing the platelets of 3 with those from the control.

Fig. 7 shows the fibrinogen levels throughout the trial. Total proteins are included for the period when fibrinogen was raised.

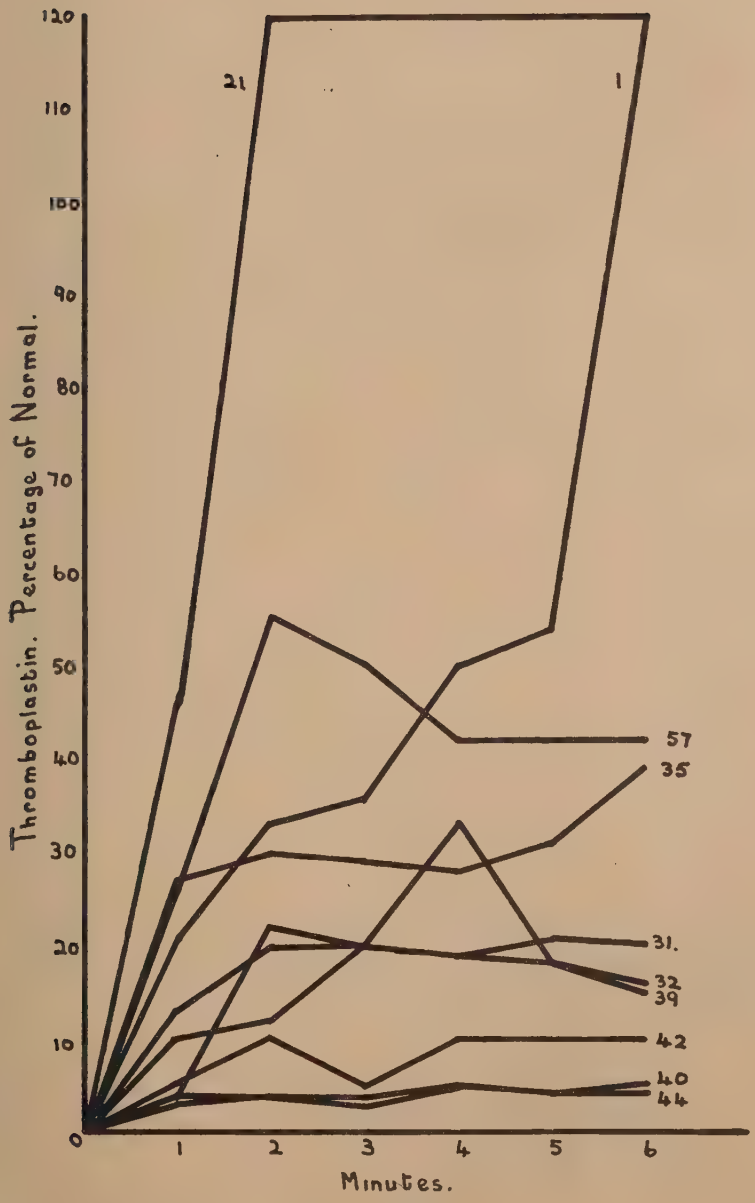
Sedimentation rate varies directly with the globulin content of the blood (Wintrobe 1951). While fibrinogen was raised the sedimentation rates rose to 5 mm. during the first hour and 8·5 mm. during the first 6 hours; sedimentation rates for the control were nil and 0·5 mm. during these times.

FIGURE 3.



Figs. 3 and 4. Figures on lines indicate day of experiment.

FIGURE 4.


120
110
100
90
80
70
60
50
40
30
20
10
0
Thromboplastin. Percentage of Normal.
1
2
3
4
5
6
Minutes.
21
1
57
35
31.
32
39
42
40
44

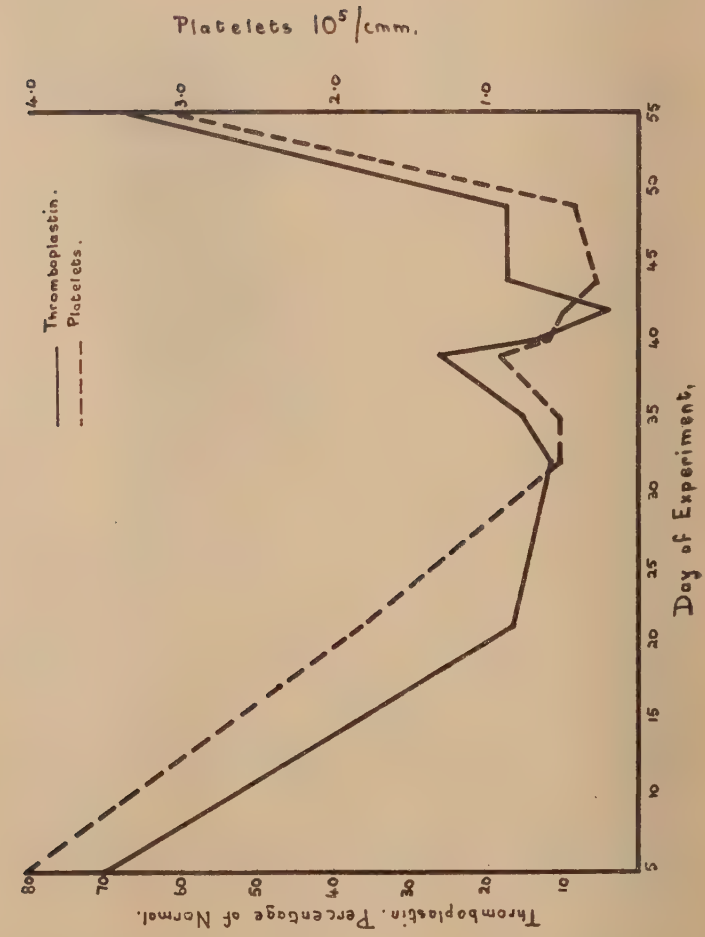
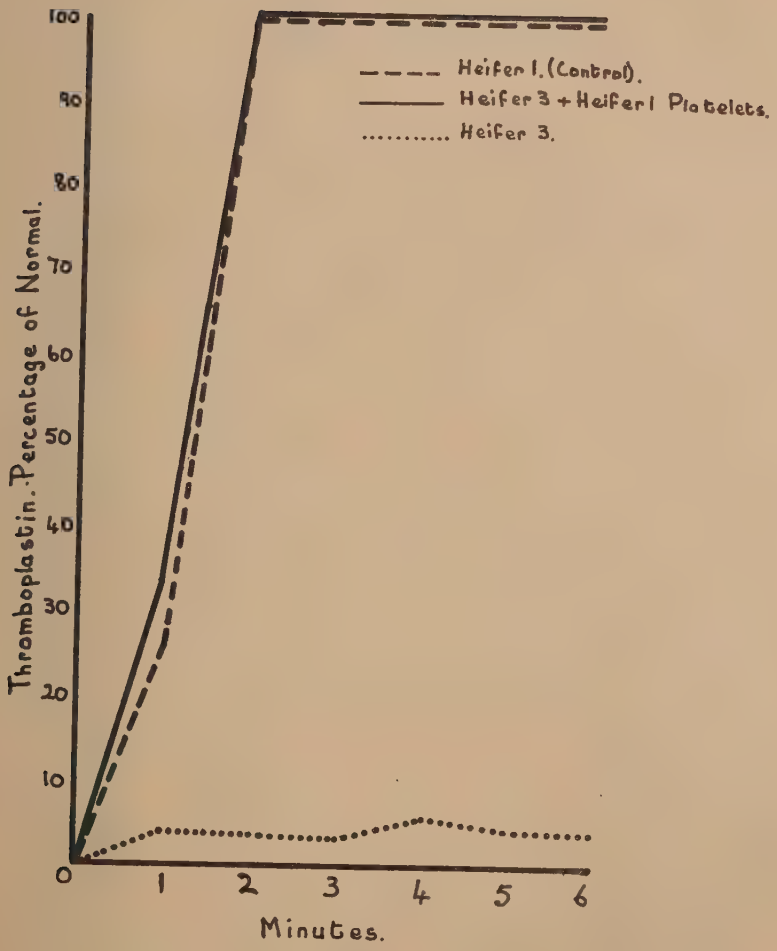

Platelets 10^5/cmm.
4·0
3·0
2·0
1·0
Thromboplastin.
Platelets.
Thromboplastin. Percentage of Normal.
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
55
Day of Experiment.

FIGURE 5.

FIGURE 6.


100
90
80
70
60
50
40
30
20
10
0
Thromboplastin. Percentage of Normal.
1
2
3
4
5
6
Minutes.
Heifer 1. (Control).
Heifer 3 + Heifer 1 Platelets.
Heifer 3.

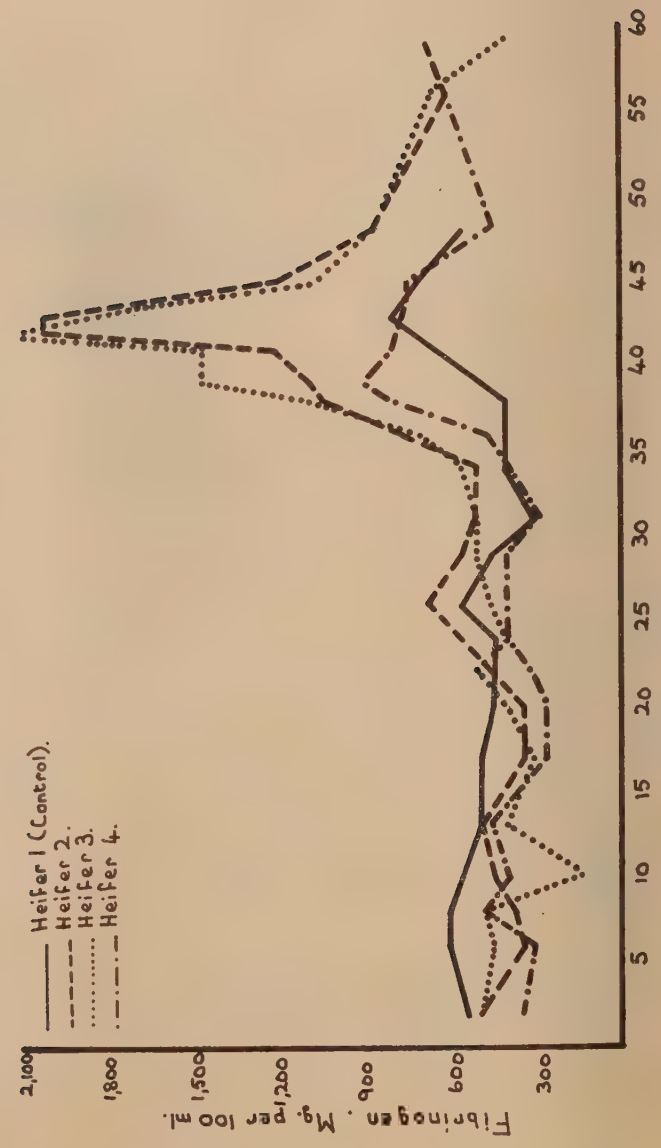

Heifer 1 (Control).
Heifer 2.
Heifer 3.
Heifer 4.
Fibrinogen. Mg. per 100 c.c.
2,100
1,800
1,500
1,200
900
600
300
5
10
15
20
25
30
35
40
45
50
55
60
Day of Experiment.

FIGURE 7.

DISCUSSION

The origin and significance of the leucopenia, agranulocytosis and thrombocytopenia have been well discussed by Evans, Evans and Hughes (1954) and Naftalin and Cushnie (1954).

The bleeding which occurs from the nostrils and into the intestine may be a result of lack of the normal process of inflammation which is associated with gross leucopenia. In radiation sickness, oropharyngeal angina and necrotic ulceration of the small and large intestines may be found (Loutit and Scott Russell, 1955) and this could account for the clinical bleeding. Prolongation of the bleeding time is more difficult to explain. Whitby and Britton (1953) state that there is no evidence to show that reduction in platelet count plays more than a secondary part in production of purpura. Zucker (1949) showed that prolongation of bleeding time in thrombocytopenia does not occur when injury is restricted to the capillaries. Our work throws no further light on this problem. It is probable that lack of the platelet clot described by Macfarlane (1956) is the factor responsible for the prolonged bleeding time, though reduction of 5-hydroxytryptamine and other platelet factors may also play some part. Since no effort was made in this work to examine qualitative defects in platelets, the matter remains one for speculation. Blood transfusion appears to be the ideal treatment.

Macfarlane and Biggs (1953) showed that above a certain minimum a reduction in the number of platelets causes a corresponding reduction in the rate of thrombin generation and in the amount of thrombin produced, but has little effect on the time at which thrombin generation begins. Reference to Fig. 2 shows that, in bracken poisoning, the time of production of thrombin remains unaltered and, for 7 minutes, rate of production is reduced but that the amount of thrombin produced exceeds normal. Since an excess of fibrinogen is used in the test this finding is not likely to be due to the increased fibrinogen content in the blood of cattle suffering from bracken poisoning. Further work will be necessary to explain this finding.

Thromboplastin generation test results reported in this paper failed to show that there is any clotting defect other than thrombocytopenia in the blood of cattle fed on bracken.

The normal level of fibrinogen in bovine blood is 700 mg./100 ml. (Duke, 1947). The level of fibrinogen in cattle fed on bracken was increased to nearly three times normal. The fact that total plasma proteins were not increased suggests that this was not due to haemoconcentration but might be a finding peculiar to bracken poisoning. It probably explains the peculiar clots which were noted in the thrombin generation tests.

CONCLUSIONS

Tests designed to demonstrate the cause of clotting defects in

cattle fed on bracken failed to show any cause other than thrombocytopenia. The production of thrombin and the level of blood fibrinogen were abnormally high; no explanation was found for these facts.

ACKNOWLEDGMENT

We wish to thank Dr. J. C. F. Poole and Dr. D. S. Robinson for their advice. We also thank Mr. J. Taylor who gave us the bracken, and Mr. P. C. Whittington for his care of the animals.

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THE PATHOLOGICAL EFFECTS OF THOROTRAST MYELOGRAPHY IN THE DOG

By

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INTRODUCTION

Thorotrast, a stabilised 25 per cent colloidal solution of thorium dioxide, has been used as a contrast medium in medical radiology since 1928. It has been the centre of much controversy over the years, and the initial enthusiasm with which it was welcomed has been tempered by the gradual recognition of its potentially dangerous nature.

Whereas in human medicine it is used mainly for the visualization of abscess cavities and occasionally in critical cases for hepato-splenography and the demonstration of vascular pathology, the medium is used in the veterinary field for myelography.

The literature on thorotrast and its uses is extensive. Reeves and Stuck (1938) give a comprehensive bibliography of the earlier work and Tesluk and Nordin (1955) enumerate more recent references. It has certain disadvantages which give it a pathological significance and have resulted in the cautious approach to its use by radiologists. During the process of decay, thorium and its disintegration products at various stages and in various proportions emit α and β particles and γ radiation. Of these, the α particles, which produce locally intense ionization, are the most important with regard to internal pathological effect. Thorium has been reported as the cause of tissue necrosis, radiation osteitis, malignant neoplasia and aplastic anaemia in man and experimental animals. Thorotrast is an irritant and is only scantily eliminated from the body. It is a colloidal preparation and when used for myelography may increase the intracranial fluid pressure causing hydrocephalus (Reeves and Stuck, 1938). Little work has been done on the pathological effects of thorotrast in veterinary radiology.

Thorotrast is most frequently used in the dog for visualizing intra-spinal conditions. Most workers inject the medium into the subarachnoid space at the cisterna magna, although lumbar puncture may also be employed. After taking the radiograph, the thorotrast may or may not be partially removed by a forced drainage technique (Nosik and Mortensen, 1938). Wustmann (1933) described the distribution of thorotrast 1073 A and D after subarachnoid injection and noted phagocytosis of the medium and sub-arachnoid round cell infiltration. Reeves and Stuck (1938) produced hydrocephalus in three dogs by ventricular injection of 1 to 2 ml. doses and recorded phagocytosis of thorotrast by macrophages in the meninges. In one experimental dog which he injected with 4 ml. of thorotrast suboccipitally, Capua (1933) found a meningitis. The dog exhibited convulsive seizures before death which occurred on the 20th day after encephalomyelography. He recorded similar findings in a human patient after lumbar injection but with milder clinical signs.

Olsson (1951) considers that the irritation of thorotrast is slight but that insignificant elimination of the material may lead to hydrocephalus and radioactive injury. He states that chemical analysis shows the cerebrospinal fluid to contain little thorotrast after one month and none at all after two months from the date of injection. He gives no pathological details. Hoerlein (1953) mentions that thorotrast can cause marked phagocytosis and fibrotic leptomeningitis. Freeman (1937) used thorotrast for ventriculography in man. He described the meningeal reaction of eight autopsied cases as consisting of an early transient leucocytic exudate followed by macrophage infiltration, and considered that the possibility of further progressive local inflammation was precluded by the inertness of thorium.

A detailed histopathological description of the lesions caused by thorotrast in the dog is lacking from the literature. This communication deals with the pathological effects of thorotrast on brain and spinal cord of 12 dogs on which myelography had been performed for diagnostic purposes.

METHODS

Sections of brain and cord together, in most cases, with routine sections of other organs were taken at post-mortem from the dogs. The dogs had been injected sub-arachnoidally via the cisterna magna with doses of thorotrast varying from 0·5 to 2·0 ml. depending upon size. The intervals between myelography and death ranged from one to 16 days. The main lesions in these cases were disc calcification and prolapse and ossifying pachymeningitis. Four cases, however, had no lesions of the brain or the cord beyond those due to thorotrast. Attention was always focussed upon sections away from the area of the main pathological lesion.

Tissues for microscopical examination were fixed in sublimate-formol or 10 per cent formol-saline, dehydrated and cleared in an alcohol-amyl acetate-benzol series and double embedded in celloidin and paraffin. Sections were stained routinely with haemalum and eosin; other techniques used on selected sections were picro-Mallory, phosphotungstic acid haematoxylin and P.A.S. Autohistoradiography was performed on some unstained mounted sections.

RESULTS

Clinical Signs

None of the dogs showed any clinical signs either immediately or at a later date after myelography, which could be related to the procedure.

Pathology

At autopsy, the cerebral and spinal meninges of all the dogs appeared macroscopically normal. Microscopical examination one day after myelography showed the subarachnoid space to contain numerous masses and plaques of thorotrast. These accumulations were of a grey, finely granular appearance and often had a slight eosinophilic tinge when stained by haemalum and eosin. This colour and the light pink staining of the masses with P.A.S. was due

to the taking up of the stain by the tapioca-dextrin associated with the thorium dioxide in the suspension. The thorotrast was highly refractile and was best seen by reducing the incident light. It was not birefringent. Under dark ground illumination it was seen as refractile silver masses. When stained by haemalum and eosin, the dark ground appearance of the thorotrast granules became bright pink. Using a Geiger-Muller counter, mounted stained sections were found to be very slightly radio-active. There was a marked cellular infiltrate into the subarachnoid space in all the cases (Fig. 1). The cells were mainly macrophages together with a small number of lymphocytes. In one case of two days duration, a large proportion of the cells were polymorphs. At first, the macrophages contained only a few granules of thorotrast, but by the end of 48 hours they had become large round or polygonal cells, greatly distended by coarse yellow-grey granules of thorotrast. The cells tended, in the first few days, to lie in great accumulations around the blood vessels in the cerebral sulci and the fissures of the cord. Sulci might be completely choked with cells and the cell borders were invisible. By the time seven to eight days had passed, most of the thorotrast had been phagocytosed, although the subarachnoid space around the optic nerve might still be packed with much free thorotrast lying in plaques and in one case, the medium was to be seen being phagocytosed outside the dura mater (epineurium). As early as two days after injection macrophages filled with thorotrast might be seen lying in the Virchow-Robin spaces of brain and cord. In this position, the medium could be found deep in the white matter of the cerebral hemispheres and cerebellum. At seven to eight days some of the macrophages had ruptured spilling their contents and many were dead or dying, showing pyknotic shrivelled nuclei. Intermingled with the other cells everywhere were red blood corpuscles and some areas showed pronounced extravasation of blood. After the first 24 hours there was a progressive increase in the amount of intercellular debris taking the form of shreds and strands of hyaline collagenous material. The adventitia of the blood vessels became frayed and collagenous shreds of the same appearance as the intercellular debris were becoming detached.

At four to five days the arachnoid membrane was slightly thickened at points by fibroblastic proliferation. This process continued, but with little deposition of collagen until the ninth or tenth day after myelography. At seven to eight days, areas of early granulation tissue surrounding blood vessels could be found in the spinal fissures where the cells were densely massed. Capillaries and fibroblasts invaded the macrophage accumulations from the periphery. By the ninth or tenth day the spinal meninges showed the most pronounced histopathological changes. The dura mater was thickened and fibrous and the collagen composing it was swollen and hyaline in appearance. The middle zone of the dura showed fragmentation of collagen fibres, infiltration with

macrophages and fibroblasts and vascularization. There was no thorotrast in this area.

The inner layers of the dura were infiltrated by thorotrast and macrophages and there was granulation of the dura which, in some places, had progressed to hyaline fibrosis. Some of the vessels of the granulation tissue showed mural fibrinoid necrosis. There were adhesions between the dura and the arachnoidea. The subdural space of the spinal cord contained a little thorotrast both free and phagocytosed. The arachnoid membrane was thickened by highly vascular granulation tissue which had developed mainly on the inner surface (Fig. 2 and 4). Much of the granulation tissue showed fragmentation of the collagen, infiltration with macrophages and thorotrast and extravasation of blood. Many macrophages contained blood pigment. Some parts of the granulating arachnoidea were expanded into fibrous nodules which frequently showed fibrinoid change (Fig. 3). The pia mater was also thickened and in places the subarachnoid space had been obliterated by granulation tissue. The pia was a thickened layer of granulation tissue containing numerous areas of collagen debris, fibrinoid necrosis and infiltration with macrophages and lymphocytes. There were some massive accumulations of lymphocytes in the pia. The surface of the pia was covered by thorotrast-containing macrophages. The meningeal vessels showed various changes depending upon size. The larger arteries were surrounded by masses of mature fibrous tissue and the smaller vessels showed fraying of the adventitia, vacuolation of the media and swelling of the intimal cells or mural fibrinoid necrosis. No vascular thrombosis was apparent, however. The lesions of the spinal meninges were pronounced throughout the whole length of the cord and extended around the roots of the spinal nerves. The cerebral meninges at nine to ten days showed an appearance similar to that found after seven days duration. Along with the subarachnoid macrophage infiltration there was mild thickening of the arachnoidea by fibroblastic proliferation. The membrane was infiltrated by thorotrast-bearing phagocytes. The microscopic picture at 16 days duration resembled that seen at nine to ten days.

At no stage could thorotrast be found in any other organ throughout the series except for one case (six days duration) where it was seen in abundance in the cervical lymph nodes (Fig. 5). The thorotrast was being phagocytosed by reticulo-endothelial cells lining the sinuses.

DISCUSSION

The histopathological study of these cases indicates the ability of thorium dioxide suspension to evoke a lepto-meningitis whose salient features are phagocytosis and remarkably rapid fibrosis. These findings are, in general, similar to those found under experimental conditions in other animals by various workers

(Radovici and Meller, 1934; Reeves and Stuck, 1938), but the lesions are more severe and rapidly progressive. The observations of Freeman (1937) on the improbability of inert thorium causing a progressive inflammation of the meninges are not true in the case of the dog. Although no clinical signs were detected in these cases which could be related to the meningitis, it would be difficult to separate such signs from the effects of the primary disease in most cases. Theoretically, one would have expected clinical signs to be evoked by the marked degree of meningeal changes found. Although myelography was performed on the dogs with all the usual precautions, the presence of thorotrast in the cerebral meninges is to be noted. It seems to be a conclusive fact (Wustmann, 1933; Radovici and Meller, 1934; Reeves and Stuck, 1938) that thorotrast is only very slightly eliminated from the subarachnoid space, even after years. This fact, together with the α particle emission of thorotrast, gives rise to speculation on the possible long term effects of the medium in the subarachnoid position. From the type of plastic meningitis produced and its probable persistence, there would appear to be a great danger of hydrocephalus. The contention of Wustmann (1933) that the intact pia forms a barrier to the penetration of thorotrast seems to be true, but the finding of thorotrast so widely deep in the Virchow-Robin spaces of the brain shows that it can carry its radioactivity throughout the central nervous system. The radioactivity of thorium may well prove to be a most important factor. The half-life of thorium is $1{\cdot}65 \times 10^{10}$ years and it is considered (Rundo, 1955) that in man, deleterious radioactive effects are likely to appear at some time after the third year following administration. In the cases which are the subject of this communication, the ionising effect of thorium was probably the cause of death of the high proportion of macrophages and also the damaging of meningeal capillaries with resultant haemorrhage. As α particles have a low penetrating power, the effects of subarachnoid thorotrast are likely to be local ones and radiation necrosis of the spinal cord, osteitis or neoplasia are possible sequelae. It is probable that subcutaneous seepage of contrast medium during injection resulted in drainage to the cervical lymph nodes and the finding of thorotrast in this position. Escape of contrast medium through the suboccipital punctures likewise accounts for the finding of thorotrast sub-durally in some cases. With other types of medium available, it is doubtful whether the use of thorotrast for canine encephalomyelography can be justified.

CONCLUSIONS

Thorotrast, which is now infrequently used in human radiology, is still used as a contrast medium in canine myelography.

The histopathological findings in the meninges of 12 dogs on which myelography had been performed for diagnostic purposes are described. The salient pathological features of the meningeal

response to subarachnoid injection of thorotrast are phagocytosis and a rapidly fibrosing leptomeningitis.

The significance of these findings is discussed.

ACKNOWLEDGMENTS

I am grateful to Mr. D. D. Lawson for some of the histological material which was drawn from a larger body of his cases, to Dr. W. F. H. Jarrett for much advice and criticism and to Mr. A. Finney for photography. This work was carried out while the author was being supported by a grant from the Agricultural Research Council for work in a comparable field.

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LEGENDS TO ILLUSTRATIONS

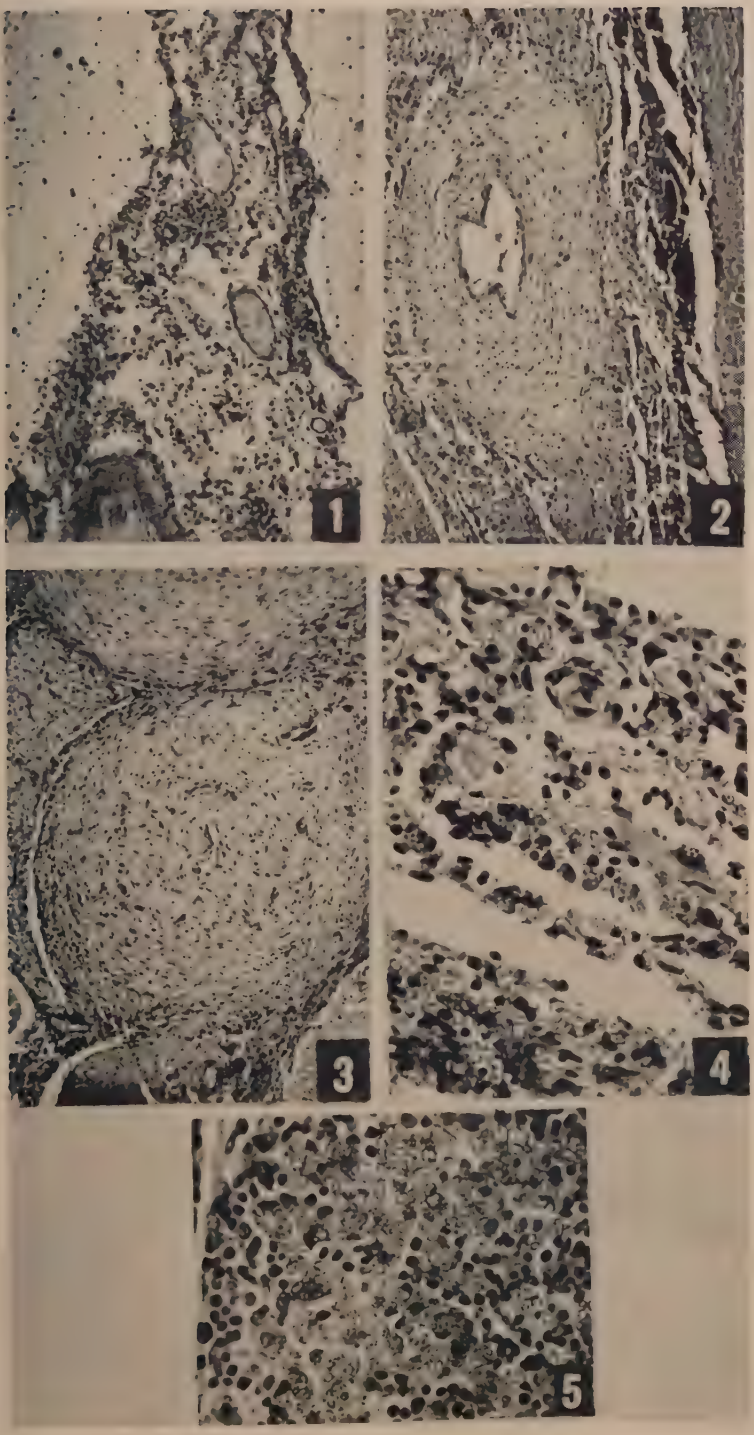
Fig. 1. Cerebral meninges two days after myelography. Subarachnoid accumulation of thorotrast-containing macrophages. H. and E. × 70.

Fig. 2. Spinal meninges. Ten days after myelography. Thickening and adhesions of the meninges produced by granulation tissue. H. and E. × 70.

Fig. 3. Spinal meninges. Ten days after myelography. Arachnoideal fibrous nodule near nerve root, showing areas of fibrinoid change. H. and E. × 70.

Fig. 4. High power view of arachnoidea in Fig. 2 showing thorotrast granules. H. and E. × 280.

Fig. 5. Middle cervical lymph node. Six days after myelography. Thorotrast granules lying in lymphatic sinuses. H. and E. × 280.



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THE RESPONSE OF THE CERVIX UTERI OF RUMINANTS TO ADRENALINE

By

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INTRODUCTION

Very little is known of the action of adrenaline on the cervix in any species. Such information as is available is confined almost entirely to laboratory animals.

Only one publication refers to domesticated animals, that of Newton (1934) who showed that isolated circular strips from the cervix of the cat suspended in Ringer's solution were stimulated by adrenaline in concentrations which failed to stimulate cornual strips from the same animal. In contrast, oxytocin stimulated the cornua but not the cervix. The same reciprocal pattern of response of the cervix and the cornua to these two drugs has also been observed in vitro in guinea-pigs (Newton, 1937) and rabbits (Bonnycastle and Ferguson, 1941). In each case adrenaline selectively stimulated the cervix and oxytocin the cornua. However, the in vivo experiments of Adler, Bell and Knox (1944) using rabbits, guinea-pigs and cats, showed that the responses of the cervix and the cornua to adrenaline were variable and that a reciprocal pattern of response, although detected occasionally, could not be obtained consistently. Schofield (1952) using the same technique in rabbits reported no difference between the cornual and the cervical responses to adrenaline, nor-adrenaline or hypogastric stimulation.

Recently in human obstetrics, sympatholytic drugs such as dihydro-ergotamine have been used to relieve cervical spasm during labour. It has been suggested by Sauter (1948) that cervical spasm is adrenergic in origin and that ergot sympatholytics in suitable dosage may antagonise this spasm and promote cervical relaxation without depressing the rhythmic contractions of the corpus. Several clinical papers have appeared indicating that dihydro-ergotamine is effective in relieving cervical dystocias (Reist, 1949; Baskin and Crealock, 1950, 1951; Gill and Farrar, 1951; Bruns, Snow and Drose, 1953 and others).

The response of the body of the uterus of ruminants to adrenaline has been described recently (Fitzpatrick, 1957a). The only previous attempts to study cervical responses to these drugs in vivo are those of Adler *et al.*, (1944) and Schofield (1952) using laboratory animals in which small recording balloons were inserted into the cervix and the cornua. The fidelity with which cervical contractions can be recorded independently of the contractions of the body of the uterus is greatly increased by using large animals: especially is this so in ruminants in which the cervical canal is distorted by several baffle-like annular constrictions which effectively screen off a small cervical balloon from both uterine and vaginal pressures (Fitzpatrick, 1957b). This is of considerable importance when the findings are interpreted in the light of the general hypothesis that the cervix has an activity independent of that of the rest of the uterus and that this is an integral part of normal physiological parturition (Fitzpatrick, 1957c).

The experiments reported below describe the responses of the cervix to adrenaline in intact cows and sheep.

METHODS

The recording apparatus and general technique were as described earlier (Fitzpatrick, 1957a) and are based on the use of small rubber balloons to measure and record intra-uterine and intra-cervical pressures. Two balloons of 1 and 2 ml. capacity respectively are mounted on a hollow 2-channel metal catheter so designed that the lumen of each balloon communicates with a separate channel in the catheter. Pressures to which the balloons are subjected during muscular contraction are independently transmitted through the catheter and appropriate connecting tubing to two recording manometers. The whole system is water-filled and can record intrauterine pressure changes of 0·5 mm. Hg. In cows the catheter is inserted per vaginam in the usual manner for uterine catheterisation and guided into position by manipulation per rectum. In sheep this guiding manipulation necessitates a temporary laparotomy. When in place the terminal (uterine) balloon is situated just caudal to the bifurcation of the uterine horns. The second (intracervical) balloon is midway along the cervical canal and this position is maintained by clipping the catheter to the vaginal protuberance of the cervix. The balloons are deflated for the catheterisation and when in position are re-inflated to two thirds of their mould volume by the injection of a predetermined volume of water into each water system. This incomplete filling enables the balloons to follow a fall as well as a rise of pressure.

All instrumentation and recordings were made under epidural anaesthesia in cows and under general anaesthesia, using cyclopropane or sodium pentobarbitone, in sheep. After inserting the instruments a period of 45 minutes was allowed to elapse before any drugs were given. Adrenaline hydrochloride solution B.P. was given intravenously by way of an indwelling polythene catheter inserted into an external jugular vein.

Terminology. For convenience, the terms "uterus" and "uterine" will be used only in reference to the whole organ including the anatomical cervix uteri. The expression "corpus" and "cervix" and derived adjectives will refer to the two parts of the organ which contain the balloons.

RESULTS

It has been shown by Fitzpatrick (1957a) that the response of uterine muscle of cows and sheep consists basically of a short stimulation followed immediately by a longer inhibition. This characteristic was found to apply also to the adrenaline induced responses of the muscle of the cervix. The intensity of the contractile and inhibitory components of the individual responses varied with the stage of reproductive activity and will be described separately for non-pregnant cows, pregnant cows and pregnant sheep. Throughout this report the variation in response of the muscle of the body of the uterus will be compared with that of cervical muscle. To facilitate quantitative expression of the magnitude of the contractile and the inhibitory components of these

responses, contraction is expressed as the maximum increase in pressure over and above that of previous spontaneous contractions, whereas inhibition is expressed as the duration of inhibition, in minutes.

Observations in Non-pregnant Cows

Responses to adrenaline were recorded at known stages of normal ovarian cycles and also from non-pregnant cows under the influence of stilboestrol.

Normal cycle. The responses of the cervix and the corpus to adrenaline were recorded simultaneously in nineteen experiments in cows at known stages of normal ovarian cycles. The nature of the cervical contractions are summarised in Table 1 from which it can be seen that variation in response is related to the stage of ovarian activity. The inhibitory component of the response was most pronounced about the time of oestrus and was absent at the height of the luteal phase. In contrast to this the contractile component was dominant at mid-cycle and became greatly reduced about the time of oestrus. The relationship between sexual activity and variation in response is shown graphically in Fig. 1.

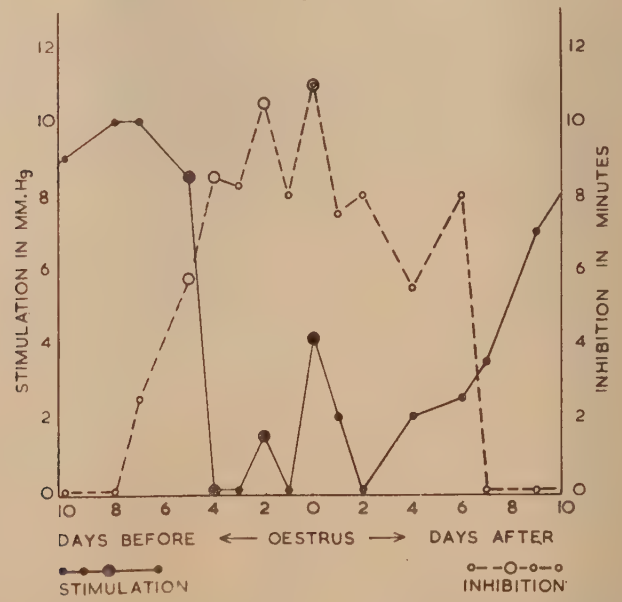
TABLE 1

RESPONSES OF THE BOVINE CERVIX TO ADRENALINE DURING THE OVARIAN CYCLE

Animal	*Stage relative to oestrus*	*Dose of Adrenaline (micrograms)*	*Increase in pressure (m.m. Hg.)*	*Duration of inhibition (minutes)*
2	12 d. prior	250	7	0
68	10 d. prior	200	9	0
16	8 d. prior	300	10	0
22	7 d. prior	250	10	2½
25	5 d. prior	200	6	6½
23	5 d. prior	250	11	5
22	4 d. prior	250	0	4½
22	4 d. prior	500	0	9½
22	4 d. prior	1000	0	10
16	3 d. prior	250	0	7½
16	3 d. prior	200	0	9
23	2 d. prior	200	2	7
23	2 d. prior	1000	4	12
22	2 d. prior	150	0	11½
22	1 d. prior	250	0	9
68	oestrus	250	0	13
22	oestrus	500	4	11
16	oestrus	250	8½	9
68	1 d. post	100	2	7½
23	2 d. post	250	0	8
23	4 d. post	350	0	5
23	4 d. post	200	4	6
68	6 d. post	250	2½	8
24	7 d. post	250	3½	0

The same sequence of response pattern is seen with the cervix as has previously been described for the corpus (Fitzpatrick, 1957a), but when the responses of the cervix and the corpus are compared with precise regard to time, the cervix shows loss of the contractile component and coincident development of the inhibitory component somewhat earlier in the cycle than does the corpus. This relationship is well illustrated in Fig. 2 in which both series of contractile responses are plotted against the stage of ovarian activity. Similarly the inhibitory responses are plotted against the stage of activity in Fig. 3. It follows that some days before oestrus the responses of the cervix may be predominantly inhibitory and that of the corpus predominantly contractile. Such contrasting responses are illustrated in Fig. 4.

Fig. 1



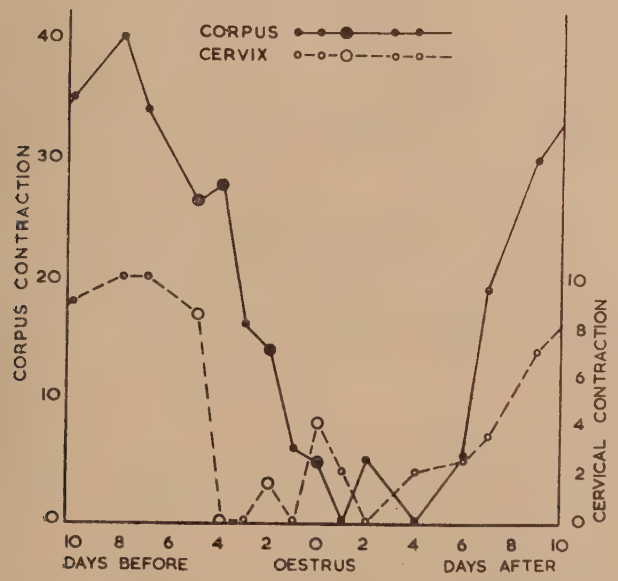
The magnitude of cervical contraction (left ordinate) and of cervical inhibition (right ordinate) after adrenaline, are plotted against the stage of the ovarian cycle in cows. Small circles: Single responses. Large circles: Means of several responses.

Oestrogen treatment. In four experiments responses of the corpus and the cervix to adrenaline were recorded before, and at varying periods after, the intramuscular administration of stilboestrol dipropionate in oil in doses known to be adequate for the production of the physiological and psychological changes characteristic of oestrus. The animals were initially in anoestrus of long duration.

In general both the cervix and the corpus showed the same

change in pattern of response; treatment with stilboestrol predisposed to an inhibitory type of response with maximal effect about the 3rd to 5th day thereafter waning. This is shown clearly in Fig. 5 in which the mean contractile and mean inhibitory responses of the cervix are plotted against the duration of treatment with oestrogen. The details of responses are given in Table 2. This change to inhibitory response under the influence of synthetic oestrogen is in keeping with the results in normal cows shortly before oestrus.

Fig. 2

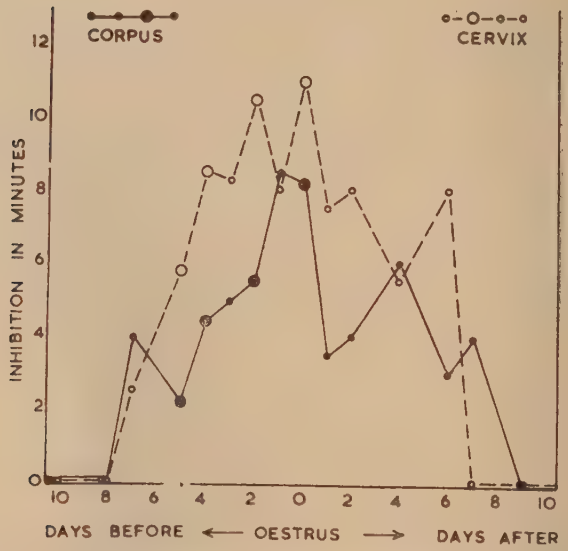


The mean contractile responses to adrenaline are plotted against stage of ovarian cycle in cows. Left ordinate: Corpus contraction in m.m. mercury. Right ordinate: Cervix contraction in m.m. mercury.

Observations in Pregnant Cows

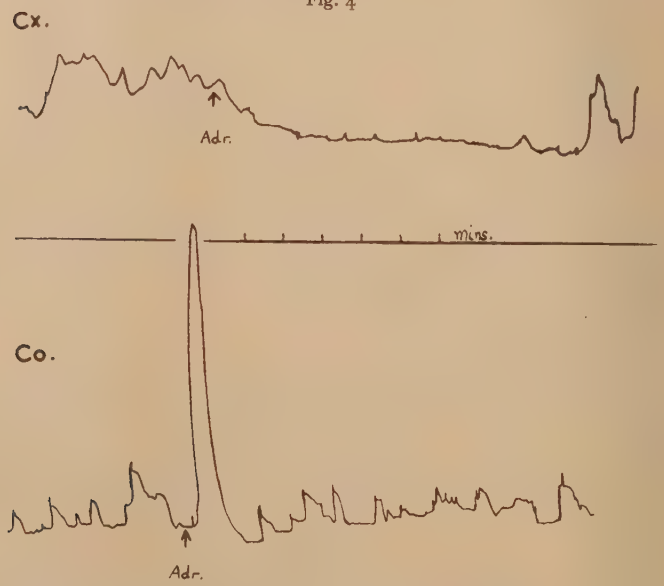
Recordings were made of cervical and corpus responses to adrenaline during the second half of pregnancy in six cows, five of which were also studied when non-pregnant. Despite repeated catheterisation, abortion occurred in only two animals, one of which was later shown to be heavily infected with *Brucella abortus*. No recordings of cervical activity can be made by this method during parturition and in the immediate post partum period, owing to the dilation of the cervical canal. The responses of the cervix are given in Table 3 and are compared with the simultaneous responses of the corpus. Inhibition of the cervix was typical of the last third

Fig. 3



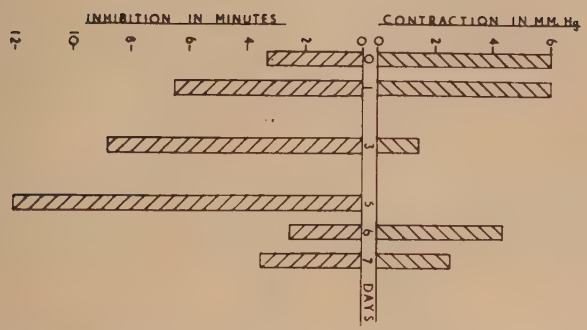
The mean duration of inhibition of cervix and of corpus, after adrenaline, is plotted against stage of ovarian activity.

Fig. 4



In a cow four days before oestrus the cervical response (above) to adrenaline is inhibitory whilst the corpus response (below) remains contractile.

Fig. 5



The contractile and the inhibitory components of the response of the bovine cervix to adrenaline are plotted against the interval following the injection of stilboestrol in oil. The values are the means of the observations in Table 2.

TABLE 2

THE EFFECT OF STILBOESTROL DIPROPIONATE ON THE ADRENALINE INDUCED RESPONSE OF THE BOVINE CERVIX

Dose of Stilboestrol	*Duration of action (days)*	*Dose of Adrenaline (micrograms)*	*Increase in pressure (m.m. Hg.)*	*Duration of inhibition (minutes)*
0	—	250	5	5
20 mg.	7	250	0	7
0	—	500	4	0
20 mg.	3	500	0	0
	5	500	0	12
0	—	250	3	0
35 mg.	3	250	0	6
	3	800	5½	8
0	—	250	12	9½
50 mg.*	1	250	6	6½
	3	200	0	18
	6	100	1½	5
	6	250	7	0
	7	250	5	0

*An initial dose of 30 mg. was followed 48 hrs. later by another of 20 mg.

of pregnancy, but contractile responses were recorded at mid-pregnancy and again for a short time close to parturition. In Fig. 6 the variation in cervix response is related diagramatically with the stage of pregnancy to show this transition from contractile to inhibitory response at about the 24th week, and the irregular reversal near parturition. The simultaneous responses of the corpus, which are almost invariably inhibitory during the second half of

pregnancy are also shown. However, isolated contractile responses of the corpus at 19 weeks and in the last week of pregnancy indicate that the same transition in response probably occurs with the corpus as with the cervix although at slightly different times. In several experiments the two responses were completely different in nature, as illustrated in Fig. 7.

TABLE 3

RESPONSES OF BOVINE CERVIX TO ADRENALINE DURING THE SECOND HALF OF PREGNANCY

Animal	*Stage of pregnancy*	*Dose of Adrenaline (micrograms)*	*Predominant response*	
			Corpus	*Cervix*
20	19 weeks	400	contract	contract
68	21 ,,	250	relax	contract
68	21 ,,	800	relax	contract
68	24 ,,	250	relax	diphasic
68	24 ,,	800	relax	contract
20	28 ,,	500	relax	relax
20	28 ,,	500	relax	relax
22	30 ,,	500	relax	relax
16	38 ,,	250	relax	relax
16	38 ,,	450	relax	relax
23	39 ,,	250	contract	contract
23	39 ,,	500	contract	contract
16	39 weeks +2 days	500	relax	relax
16	39 weeks +4 days	250	relax	relax
23	39 weeks +5 days	500	relax	contract
16	39 weeks +6 days	100	relax	relax

Observations in Pregnant Sheep

Responses to adrenaline were recorded in 12 experiments in the second half of pregnancy, each animal being studied once only. Four observations were also made between the 3rd and 5th days after parturition. The character of the cervical responses is summarised in Table 4 from which it can be seen that contractile

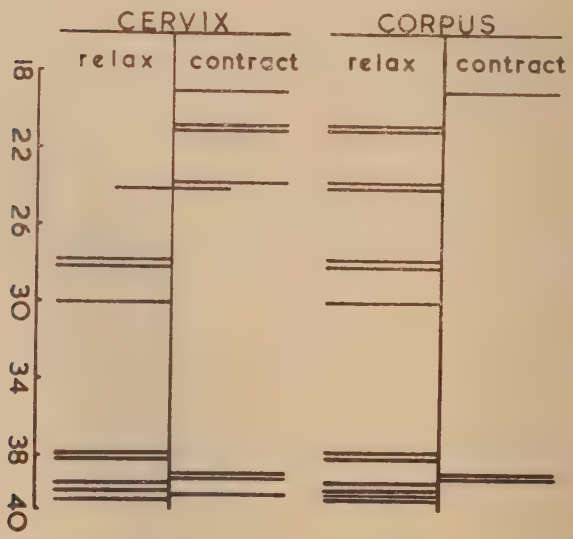
responses were obtained most frequently and pure inhibitory responses were recorded for only a relatively short period towards the end of pregnancy. In the post-partum period the responses revert to the contractile type. The corresponding corpus and cervix responses are compared diagramatically in Fig. 8 which should be compared with Fig. 6. In the sheep as in the cow there is a transition from contractile response at mid-pregnancy to inhibitory response in later pregnancy and this is seen with both the cervix and the corpus. In both species the inhibitory type of response appears sooner and more completely in the corpus, the cervix retaining the contractile component of response until later.

TABLE 4

RESPONSES OF THE CERVIX OF THE SHEEP TO 100 MICROGRAMS ADRENALINE DURING THE SECOND HALF OF PREGNANCY

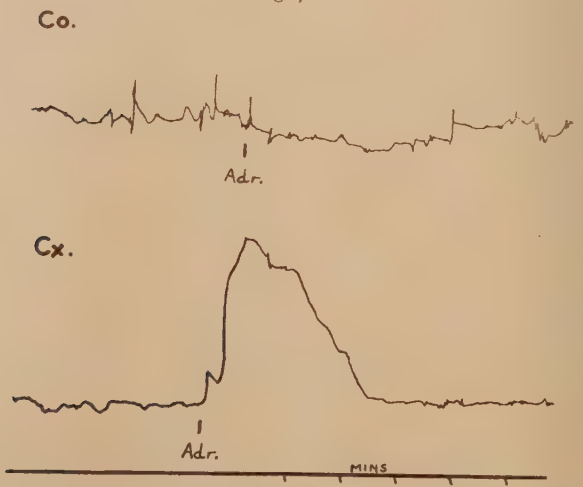
Stage of pregnancy	*Predominant response*	
	Corpus	*Cervix*
11 weeks	diphasic	contract
12½ „	contract	contract
13 „	contract	contract
14 „	diphasic	contract
14 „	relax	contract
15 „	relax	contract
15 „	relax	diphasic
16 „	relax	contract
17½ „	relax	contract
18½ „	relax	contract
19 „	relax	relax
20 „	relax	relax
20 „	relax	relax
21 „	contract	relax
3 days post part	diphasic	relax
4 „ „ „	contract	contract
4 „ „ „	contract	contract
5 „ „ „	contract	contract

Fig. 6



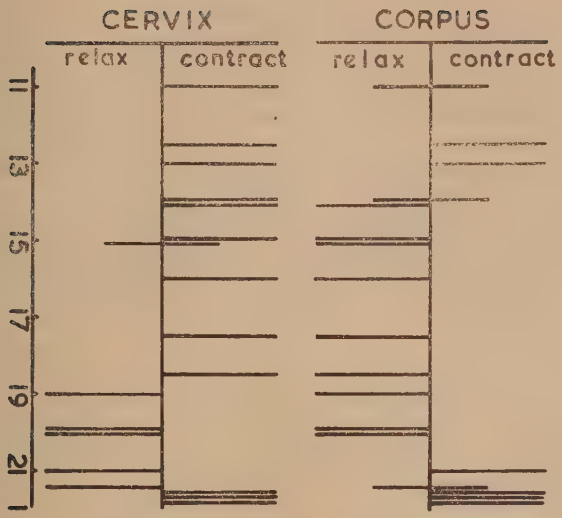
The nature of the corpus and cervical response to adrenaline is plotted diagramatically against duration of pregnancy (in weeks in the cow. In each case pure contractile responses are shown above a horizontal axis, and pure inhibitory responses below, with mixed responses occupying an intermediate position.

Fig. 7



At mid-pregnancy in the cow the cervix (Cx) response to adrenaline is contractile and that of the corpus (Co) inhibitory.

Fig. 8



At mid-pregnancy in the sheep the cervix (Cx) response to adrenaline is contractile and that of the corpus (Co) inhibitory.

DISCUSSION

In the non-pregnant bovine the response of the cervix to adrenaline is similar to that of the corpus. Both structures in general respond with contraction when under the influence of progesterone and with relaxation under the influence of natural and synthetic oestrogens. Although the responses of the cervix and the corpus are affected qualitatively in the same way by ovarian hormones, it does not follow that they are invariably similar at all stages of the cycle. It was found that during a brief transition phase at about the 17th day of the cycle the cervical response lost its contractile component and changed to inhibition when the corpus might still be contracting powerfully. Presumably this distinction between the cervix and the corpus is a reflection of differing sensitivities of the two structures to the prevailing sex hormones.

Observations on sheep and cows over the second half of pregnancy show that in both species the response of the cervix to adrenaline is more contractile than that of the corpus. This, however, is complicated by a pattern in time by which the reaction of both the cervix and the corpus tends to become increasingly inhibitory as pregnancy progresses. The change from predominantly contractile to predominantly inhibitory responses does not occur simultaneously in the corpus and the cervix, presumably because of differing sensitivities to variations in progesterone and oestrogen

balance. In sheep there was good evidence that over a period of several weeks the two structures responded dissimilarly, the corpus showing inhibition and the cervix contraction. In bovines the change from contraction to inhibition during pregnancy was seen more clearly with the cervix. The reaction of the corpus had apparently already passed to inhibition by the twenty-first week of pregnancy and contraction was only represented in a single experiment at 19 weeks—the earliest stage available for examination. It is probable that had it been possible to examine cows at an earlier stage of pregnancy, contractile responses would have been recorded. In the period immediately prior to parturition the responses became less consistent and some degree of reversal to contraction was seen. This is not surprising in view of the variation in sex hormone secretions at this time.

These results are of interest in relation to earlier work. The view that the muscle of the cervix may contract independently of the corpus (Newton, 1934; Bell, 1952; Fitzpatrick, 1957b) is substantiated by these experiments in which the two structures showed reciprocal polarity at certain times. Observations in ruminants in comparison with those in laboratory animals are of particular value in this field due to the confidence with which recordings of cervical activity can be attributed to true cervical contractions, without risk of mechanical transmission of pressure from the corpus or the vagina.

Earlier workers (Newton, 1934; 1937; Bonnycastle and Ferguson, 1941) using in vitro methods, have suggested that differences in reactivity to adrenaline are absolute i.e. that cervical muscle contracted and cornual muscle relaxed with adrenaline. More recent in vivo observations, using a variety of drugs (Adler *et al*, 1944; Fitzpatrick, 1957b) indicate, however, that the two structures behave according to a common pattern and that differences between them are often quantitative rather than qualitative. The present observations make both these viewpoints acceptable physiologically, in that they substantiate the existence of general similarities of behaviour of the cervix and the corpus, and indicate that the dramatic differences found by Newton in the goat and Bonnycastle and Ferguson in the rabbit are seen only for a relatively restricted period in late pregnancy and are probably associated with changes in sex hormone secretion.

Variations in the balance of sex hormones occur at other times and may be associated with differing responses of the corpus and the cervix as occurred in our non-pregnant cows. Thus, the very great fluctuations in oestrogen and progesterone secretion that occur at parturition may well represent a physiological mechanism for modifying the responses of the cervix and the corpus to sympathetic discharge and thus for controlling the process of parturition, as suggested by Ferguson (1941).

CONCLUSIONS

The action of adrenaline on the muscle of the cervix of sheep and cows has been studied with a cannula recording intra-uterine and intra-cervical pressures concurrently. The response of the cervical muscle is characteristically a short contraction followed by a longer inhibition.

Sex hormones appear to produce similar variation in the character of the cervical response as for the response of the corpus. However, the sensitivity of corpus and cervix to this action of sex hormones is dissimilar, so that at specified stages of reproductive activity responses of the two structures to adrenaline are completely independent and may even be in opposition.

The results of these experiments are interpreted in relation to earlier work.

ACKNOWLEDGMENTS

The author is grateful to Dr. H. O. Schild of University College, London, for his assistance in interpreting some of the results, and to the London Rubber Company for the gift of a generous supply of specially moulded latex-rubber balloons.

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THE EXPERIMENTAL PRODUCTION OF STAPHYLOCOCCAL MASTITIS IN THE GOAT

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INTRODUCTION

This paper describes a method for the experimental production of staphylococcal mastitis in the goat and records the observations made on the course of the disease produced. The gross post-mortem lesions and histopathology of the condition will be described in a future paper. This work constitutes the first stage of an investigation into the possibility of using vaccination to help in the control of bovine staphylococcal mastitis, in which it is intended to use goats for preliminary testing of vaccines.

MATERIALS AND METHODS

Goats. The goats used were two-year old animals from the Compton herd, in the first month of their first lactation, housed in loose-boxes and hand milked twice daily. They received a constant diet throughout the experiment.

Strain of Staphylococcus used for infection. Strain 201 used in these experiments was isolated originally from a naturally occurring case of bovine mastitis. It was a typical *Staphylococcus aureus*.

Clinical examination. Rectal temperatures were recorded twice daily from four days prior to inoculation and a general clinical examination of the goats made at least once daily. Particular attention was paid to the udder and to the physical appearance of the milk.

Observations on milk. The milk yield from each side of the udder of each goat was recorded twice daily from seven days prior to inoculation. Samples of fore-milk were collected aseptically twice daily following inoculation of the udders and on the day prior to inoculation. Three consecutive morning samples were collected during the week before inoculation. Each sample was subjected to examination as under.

Bacteriological examination. After mixing, 0·02 ml. amounts of milk were spread on Bacto-tryptose ox blood agar plates which were incubated at 37°C for 24 hours, after which time the number of colonies was counted. Colonies morphologically resembling *Staph. aureus* were identified by a gel diffusion plate precipitin test, in which an acid extract of the strain isolated from milk was compared with an extract of strain 201 in adjacent wells, against a 201 anti-serum prepared in rabbits (Pattison and Matthews, 1957).

Neutrophil count. The modified Breed smear technique described by Pattison and Holman (1951) was employed.

Whiteside test. This was performed as described by Howell, Pattison, Holman and Smith (1954).

pH determination. This was carried out on morning samples only using a pH meter.

Blood leucocyte counts. Total leucocyte counts and differential counts were performed on fresh jugular blood on three consecutive days during the week prior to inoculation, on the day before inoculation, several times on the day of inoculation and daily thereafter until the end of the experiment.

Preparation and administration of inocula. Cultures of *Staph. aureus* 201 were grown in Bacto-tryptose glucose glyceryl phosphate broth (Pattison and Matthews, 1957), and the following inocula used: (a) Sterile Bacto-tryptose glucose glyceryl phosphate broth; (b) living, 18 hour whole culture; (c) living cells, obtained by centrifuging 18 hour whole culture and resuspending to the original volume in sterile broth; (d) 18 hour whole culture killed by heating in a water bath at 100°C for 30 minutes; (e) killed cells, obtained by centrifuging whole culture killed as in (d), and resuspending the deposit in sterile broth; (f) filtrate of 18 hour whole culture, prepared by filtering through a Chamberland L2 filter candle under 300 mm. Hg pressure.

Viable counts were performed on living inocula by surface plating of saline dilutions on Bacto-tryptose ox blood agar. Control inocula were shown to be sterile by heavy plating on the same medium. The inocula were administered into the teat canal as described by Pattison, Taylor and Holman (1950). Only the right side of the udder was inoculated.

Plan of experiments. The investigation was conducted in three experiments (Table 1). In the first experiment the effect of inoculating 10 ml. of whole culture was determined on four goats, and in the second this effect was confirmed and compared with that produced by the control inocula as shown in the Table. In the third experiment the effect of inoculating progressively smaller doses of living organisms was determined. Post-inoculation observations were limited to a period of up to nine days, some goats being slaughtered before this time either on humane grounds or to provide material for histological examination. After slaughter, cultures were made from left and right supramammary lymph nodes and from the spleen of each goat by plating on Bacto-tryptose ox blood agar.

RESULTS

Reaction to Control Inocula

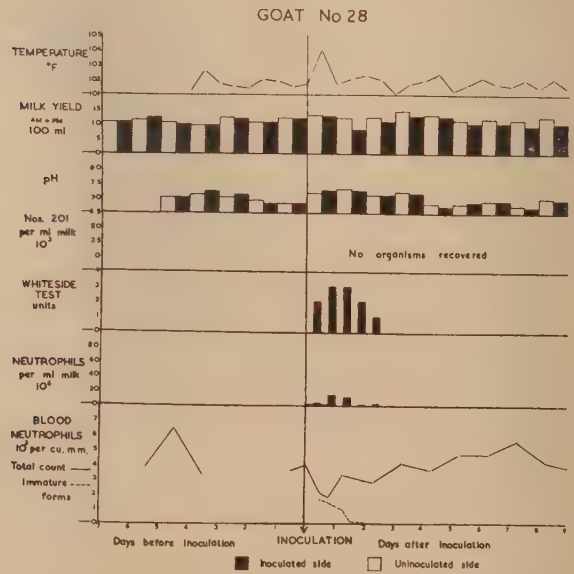
Sterile broth. The only detectable abnormality produced by the inoculation of 10 ml. sterile broth was the presence of very small numbers of neutrophils in the milk of one case, 24 hours after inoculation.

Killed whole culture, killed cells in broth and Chamberland L2 filtrate. These inocula produced similar reactions. The observations made on one of the goats, representative of the series, are shown in Fig. 1. In general the reactions resembled those of animals inoculated with living staphylococci, but were much less marked, and more transient.

Between three and six hours after inoculation there was a mild transient febrile reaction and the inoculated side of the udder was slightly swollen. Twenty-four hours after inoculation the goats were clinically normal. There was a slight, transient fall in milk yield from the inoculated side, the milk containing moderate numbers of normal neutrophils with a positive Whiteside reaction of

up to 3 units for two days after inoculation. There was no constant change in the pH of the milk and no living organisms were recovered. No changes were demonstrated in the non-inoculated side of the udder. Neutropenia rapidily developed after inoculation and was quite marked, with a shift to the left, at 6 hours. The count had returned to normal 18 hours later, and the shift to the left did not persist beyond the second day after inoculation. At post-mortem cultures from the supra-mammary lymph nodes and spleen were negative.

Fig. 1



Reaction to the inoculation of 10ml. killed whole culture into the right teat canal.

Reaction to Living Inocula

The inoculation of living Staphylococci into the udder produced mastitis in all cases except one (Goat 26) in which the dose of organisms was very small. For the purpose of description it is convenient to classify the types of reaction into: (a) gangrenous, (b) severe, non-gangrenous, (c) mild. Details of the reactions of the various goats inoculated with living organisms are given in Table 1.

Gangrenous mastitis. This type of reaction was characterised by the rapid development of gangrenous changes in the udder accompanied by a severe, prolonged systemic disturbance. Details of the observations made on one goat which developed gangrenous mastitis are shown in Fig. 2.

A typical marked febrile syndrome developed within six hours of inoculation when the dose of living organisms was high, but this was delayed for up to 24 hours when the dose was reduced. As the body temperature rose, the inoculated side of the udder became hot, swollen, tense and painful and within 24 hours showed purplish skin discolouration extending dorsally from the base of the teat. The gland later became almost black in colour and cold to the touch by which time the body temperature was normal, or sub-normal. Corresponding with the skin discolouration, the secretion from the infected side became watery and heavily blood-stained, and subcutaneous oedema extended anteriorly along the right side of the floor of the abdomen as far as the costal arch. Of the eight goats which showed this gangrenous change, seven had to be slaughtered on humane grounds within three days of inoculation at which time the animals were comatose. The eighth goat was slaughtered for histological examination eight days after inoculation.

TABLE 1

PLAN OF EXPERIMENTS

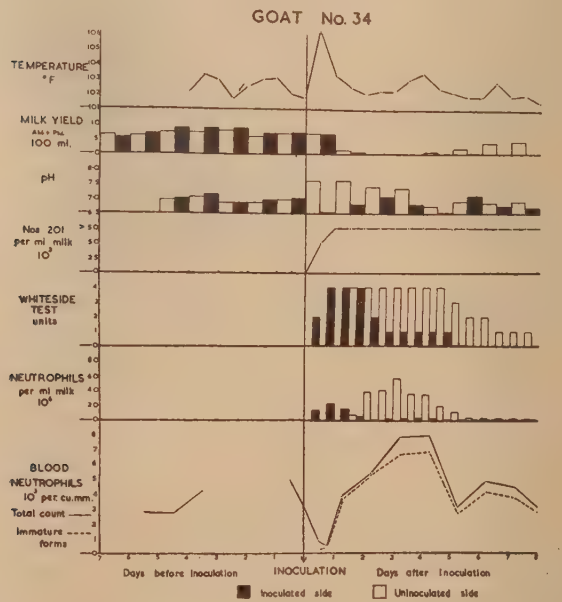
Experiment No.	*Goat No.*	*Inoculum*	*No. of living organisms in inoculum*	*Period of observation*	*Type of mastitis produced by living inocula*
1	1	Whole culture	10^9	9 days	Severe non-gangrenous
	2	,, ,,	,,	1 day	Gangrenous
	3	,, ,,	,,	2 days	,,
	4	,, ,,	,,	,,	,,
2	24	Whole culture	10^9	9 days	Severe non-gangrenous
	33	,, ,,	,,	1 day	Gangrenous
	22	Living cells	,,	2 days	,,
	25	,, ,,	,,	,,	,,
	21 and 35	Broth	Nil	2 and 9 days	None
	28 and 29	Killed whole culture	,,	,,	,,
	27 and 23	Killed cells	,,	,,	,,
	32 and 36	Filtrate	,,	,,	,,
3	26	Diluted whole culture	10^8	8 days	Infection not established
	30	Diluted whole culture	10^5	3 days	Gangrenous
	37	Diluted whole culture	10^7	8 days	Mild
	34	Whole culture	10^9	8 days	Gangrenous

In each case the inoculum used was 10 ml. injected into the right teat canal only.

The yield of milk from the inoculated side of the udder fell to nil within 24 hours of the temperature rise and never increased again. There was an equivalent fall in the yield from the uninoculated side, but in Goat 34 the yield began to increase again

five days after inoculation. At each examination after inoculation very large numbers of *Staph. aureus* strain 201 were present in the udder secretion and no fluctuation was detected. In goat 34, from the sixth to the eighth day large numbers of an organism morphologically resembling *Clostridium welchii* were isolated from the right udder secretion on Bacto-tryptose ox blood agar plates incubated anaerobically. These organisms were also seen in Breed smears of milk from Goat 34 from the sixth day onwards. No bacteria were detected in milk from the uninoculated gland of any goat.

Fig. 2



Gangrenous mastitis produced by the inoculation of 10^9 living *Staph. aureus* strain 201 into the right teat canal.

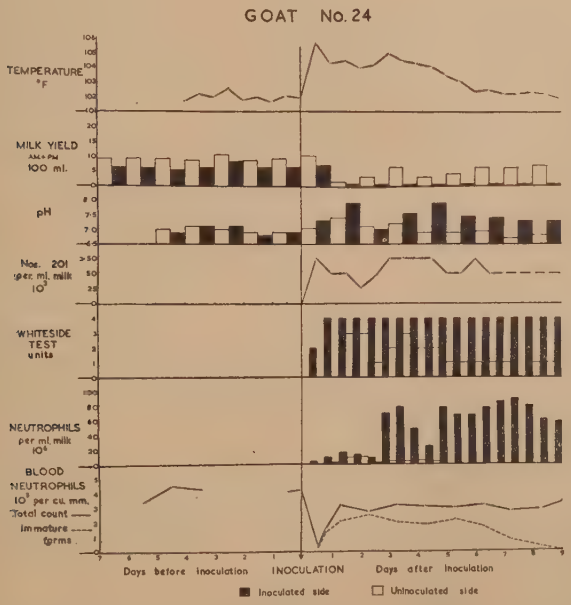
Large numbers of normal polymorphs were found in Breed smears from the inoculated side for 24 to 48 hours after inoculation, but the numbers decreased as gangrenous changes developed at which time the blood-stained watery udder secretion contained only small numbers of degenerate polymorphs and epithelial cells. Normal polymorphs were detected in Breed smears of milk from the non-inoculated gland when the yield from this gland was very low. The Whiteside test gave a close correlation with the milk neutrophil count. There was a strong positive reaction when the secretion contained large numbers of neutrophils and a negative reaction later in the disease when neutrophils were absent from the blood-

stained watery secretion. Determination of the pH of milk samples gave variable results which showed no constant abnormality.

The blood neutrophil count fell sharply to a low level when the temperature rose, but the count began to rise again 24 hours later, even in those cases which had to be slaughtered in extremis. In Goat 34 there was a phase of neutrophilia following the initial neutropenia. Within 24 hours after inoculation the neutrophil count consisted almost entirely of immature forms and these persisted in large numbers to the end of the experimental period. At post-mortem, bacteriological cultures from the spleen were negative in all cases, but small numbers of *Staph. aureus* strain 201 were recovered from the supra-mammary lymph node of one goat only (No. 30).

Severe non-gangrenous mastitis. This type of reaction, depicted for Goat 24 in Fig. 3 was characterised by an initial, systemic disturbance and very severe mastitis, but without gangrenous changes in the udder.

Fig. 3



Severe, non-gangrenous mastitis produced by the inoculation of 10^9 living *Staph. aureus* strain 201 into the right teat canal.

A marked febrile syndrome followed within six hours of inoculation and the udder became hot, swollen, tense and painful. The fall in temperature after the initial rise was more gradual than in gangrenous mastitis and Goat 24 showed a further transitory rise

of one degree on the third day after inoculation. The uniform swelling of the udder gave place to discrete palpable indurations and five days after inoculation the right supra-mammary lymph node was palpably enlarged. Milk from the inoculated gland contained large clots and was later sero-purulent in nature.

The yield of milk from both sides of the udder fell to a very low level within 24 hours of inoculation and remained low from the inoculated side. The yield from the opposite side returned to its pre-inoculation level as the general condition of the goat improved. *Staph. aureus* strain 201 was recovered in very large numbers for two days after inoculation and then in decreasing numbers in Goat 1. The numbers recovered from Goat 24 remained high throughout the period, showing a marked increase corresponding with the secondary temperature rise on the third day. Bacteria were not isolated from the non-inoculated glands.

Breed smears differed from those of gangrenous mastitis in that the neutrophil counts were higher and fewer neutrophils were degenerate. The milk neutrophil count of Goat 24 increased with the secondary temperature rise. The number of epithelial cells in Breed smears was low. As in the case of gangrenous mastitis neutrophils were present in the milk from non-inoculated glands giving a very low milk yield. The Whiteside test gave a good correlation with milk neutrophil counts. As in gangrenous mastitis, the pH of the milk did not give a clear indication of the presence of mastitis.

A neutropenia with shift to the left rapidly developed after inoculation and this was as pronounced as in gangrenous mastitis. The count returned to normal again within 24 hours, although immature neutrophils were present for several days, but had disappeared by the end of the experimental period. At post-mortem cultures from the lymph nodes and spleen were negative.

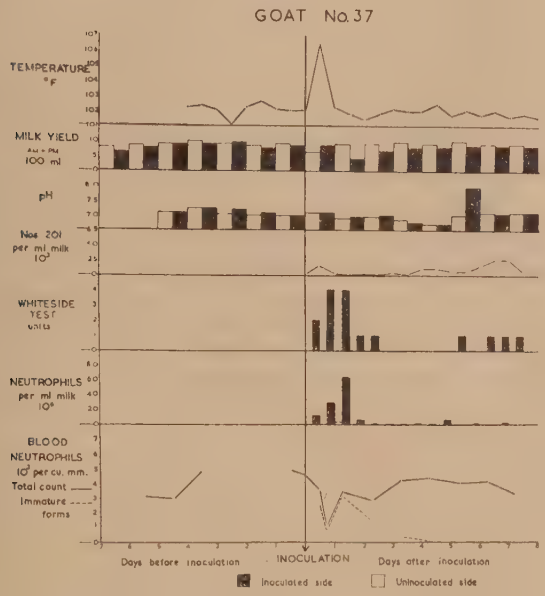
Mild mastitis. A mild type of reaction was shown by one goat (No. 37) and was characterised by an initial transient febrile reaction followed by mild inflammation of the inoculated side of the udder (see Fig. 4).

Within six hours of inoculation there was a temperature rise of four degrees and the udder was swollen and painful. This initial change was less severe than in gangrenous and severe mastitis, however, and the condition of the goat had returned to normal in 24 hours.

The yield of milk from the inoculated side of the udder was reduced by 50 per cent after inoculation, but had returned to its previous level within four days. The yield from the non-inoculated side fell during the febrile response. *Staph. aureus* strain 201 was recovered at each milk sampling following inoculation, but in smaller numbers than in gangrenous and severe reactions. No organisms were isolated from the non-inoculated side of the udder.

For two days after inoculation the number of neutrophils in Breed smears was high; the cells were always normal in appearance. The neutrophil count was paralleled by reactions given to the Whiteside test. No constant pH changes in the milk were recorded.

Fig. 4



Mild mastitis produced by the inoculation of 10^7 living *Staph. aureus* strain 201 into the right teat canal.

Following infection, the blood neutrophil count was reduced to 25 per cent of its pre-inoculation level and as in the other types of reaction, adult polymorphs were replaced by immature neutrophils. The pre-inoculation level was restored on the following day and immature neutrophils were found only occasionally after the second day after inoculation. At post-mortem cultures from the supra-mammary lymph nodes, and from the spleen were negative.

DISCUSSION

The types of mastitis produced experimentally in the goat by this strain of *Staph. aureus* resembled naturally-occurring staphylococcal mastitis in cattle and sheep. They were also similar to experimental bovine cases which have been reviewed by Klastrup, (1956) and to the experimental disease produced in sheep by Maclay, Rankin, Loosmore and Slavin (1946). The most striking

difference between the disease described in this paper and experimental streptococcal mastitis in the goat (Pattison and Holman, 1951; Holman, Smith and Pattison, 1953) was the production of gangrenous changes by staphylococci. In the present work, the rise in temperature on the third day after inoculation occurring in Goat 24, with the corresponding rise in milk neutrophil and bacterial counts, was the only evidence that what was described as a "crisis" by Holman, Pattison and Gordon (1952) may occur in experimental staphylococcal mastitis. The fact that *Staph. aureus.* strain 201 was only recovered from one supra-mammary lymph node and never from the spleen indicates that the disease is localised within the udder: in addition, there was never evidence of spread of infection to the non-inoculated side of the udder.

The isolation of an anaerobic organism resembling *Cl. welchii* from the udder secretion of Goat 34 does not suggest that this organism was of primary importance, since gangrenous changes were obvious before it was detected. Minett (1937) isolated *Cl. welchii* from two out of seven cows with gangrenous mastitis, but was of the opinion that the organisms were not primarily responsible for the changes. Parshall (1934), however, on the basis of field cases in California and a limited series of experimental cases, suggested that anaerobes were important in the production of severe gangrenous mastitis.

Experiment 3 has shown that the type of reaction produced by the inoculation of living staphylococci into the udder does not depend entirely on the dose of organisms although the highest dose always produced a severe reaction. The only constant effect of reducing the dose appeared to be to increase the length of time between inoculation and the occurrence of a detectable reaction.

CONCLUSIONS

Mastitis was produced in eleven out of twelve goats inoculated into the teat canal of one side of the udder with varying numbers of living *Staph. aureus* strain 201. Inoculation was followed by a systemic reaction and local udder changes which varied from mild inflammation to rapidly developing gangrene.

The inoculation of sterile broth into the teat canal of two goats produced no systemic reaction, and only minimal changes in the milk. Heat-killed staphylococcal cultures, heat-killed staphylococci suspended in broth and Chamberland L2 filtrate of whole culture, each inoculated into two goats, produced a transient systemic reaction and slight abnormalities in the milk.

A marked neutropenia with shift to the left rapidly developed after the inoculation of living staphylococci and a similar, but less marked change, occurred when killed culture or filtrate was inoculated. *Staph. aureus* was never recovered from the spleen after death and was recovered from the supra-mammary lymph node of one goat only, killed in extremis three days after inoculation.

ACKNOWLEDGMENT

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THE RED CELL ANTIGENS OF THE HORSE

Antigens Defined by Naturally Occurring Horse Isoantibodies

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INTRODUCTION

In an earlier paper on haemolytic disease of newborn thoroughbred foals, Coombs, Crowhurst, Day, Heard, Hinde, Hoogstraten and Parry (1948) commented on the need for a complete serological study of the blood groups in the horse with the view, amongst others, to the selection of compatible blood for transfusion of affected animals. Panisset and Verge (1922) reported that the reactions between samples of horse cells and serum were so infrequent that it was not possible to group the blood as in the case of man and unless an animal had received previous transfusions, cross-matching of donor and recipient blood was unnecessary. These authors were criticised by Hirszfeld and Przesmycki (1923) who maintained that the pattern of reactions between samples of horse cells and serum revealed 3 distinct groups—named A, B and C but gave no details of the method by which they established the likeness of these groups to the human ABO system. Landsteiner and van der Scheer (1924) also reported that 40 samples of horse blood fell into these 3 main groups. Both teams agreed that many samples of horse serum lack the expected agglutinins, e.g. Hirszfeld and Przesmycki stated that 60 per cent. of group B animals contained anti-A in their serum and only 10 per cent. of group A contained anti-B. All 4 groups, however, i.e. A, B, AB, and O, were defined by Dujarric de la Riviere and Kossovitch (1930), who also reported that the agglutinins anti-A and anti-B were missing in 20 per cent of cases studied. The groups which they described were not correlated with the human blood grouping system.

Fourie (1935) attempted to group horse bloods by completing a "chequer board" table of the reactions between cells and sera. He found that 60 per cent of the cell samples could be grouped as 'AB', and 6 per cent as group 'O', but that the remainder of the cell reactions did not fit into the ABO pattern. Schermer, (1928, 1934), Schermer, Hofferber and Kaempffer (1932) and Schermer and Kaempffer (1933) classified horse blood samples according to the presence or absence of 6 cell factors, A, B, C, D, E and F and their corresponding iso-agglutinins. Schermer (1928) also reported the occurrence of four groups, A, B, AB and O "as for man" with the proviso that the serum did not always contain the expected agglutinins. Owing to the lack of published details on the techniques, it is impossible to say whether the groups named 'A' and 'B' by these authors were considered by them to be directly related to the human ABO system.

Walsh (1924), Guermaine (1936) and Herman (1936) attempted to clarify by absorption methods the identity of the agglutinins present in samples of horse serum. Walsh noted a definite pattern in a series of reactions between horse cells and sera and formulated the theory of the

existence of at least two separate iso-antibodies. He attempted to separate and define them by selective absorptions and was not successful. A study of his protocol suggests that absorption with any chosen cell was not complete, thus invalidating the results of the post-absorption tests. Apart from species-specific agglutinins, Guermaine claimed that horse serum could be grouped according to whether or not it possessed agglutinins reacting with human A and/or B cells. This claim was established on the grounds of absorption of samples of horse sera with human group O cells and the subsequent reactions of the absorbed sera with human A and B cells. He did not test horse cells for the presence of A and B antigens. Herman, however, concluded from a series of intraspecies absorption experiments that not only could horse serum be shown to contain agglutinins for human A and B cells but that horse cells could be grouped with human isoagglutinins, anti-A and anti-B.

It is not clear from these various references whether the interaction of samples of horse cells and serum is due only to a system similar to the human ABO system; for example, Herman states that he was not able to group 5 per cent. of his samples under these conditions. The present study attempts to define the naturally occurring isoagglutinin-antigen system in the horse and to determine whether it is related to the human ABO system.

MATERIALS AND METHODS

Animals

The horses from which blood samples were taken included 41 of a herd of Exmoor, Shetland and Welsh Mountain ponies and various crosses between these, maintained and bred on the farm, 5 thoroughbred mares, a thoroughbred filly, a thoroughbred colt and 4 geldings. Each animal was named and could be recognised by the stockman and the author.

Collection of Blood Samples

Serum. The animals were bled, with the usual aseptic precautions, from the external jugular vein into a dry sterile bottle and the serum allowed to separate from the clot for 24 hours at room temperature. The serum was then separated by centrifugation and stored at —20°C. Before being used in any test it was heated for 30 minutes at 56°C. to destroy complement activity.

Cells. The ponies were bled in the same way, either into 1.2 ml. of 2.5 per cent Sequestrene (the disodium salt of ethylene diamine tetra-acetic acid) per 10 ml. of whole blood, or into 2.85 ml. of acid citrate dextrose (A.C.D.) containing 2.73 g. glucose + 2.37 g. disodium hydrogen citrate in 100 ml. of distilled water per 10 ml. of whole blood, autoclaved at 15 lb. for 30 minutes. The samples were stored at + 4°C.

The A.C.D. was found to be the better anticoagulant for this work; little haemolysis or deterioration of the samples took place even when they had been stored for 14 days. The cells were washed 3 times in 0.9 per cent. saline and made up to 2 per cent. suspensions for use. Fresh cell samples were prepared in this way for each day's work.

Collection of Saliva Samples

Sterile cotton wool swabs held by a large pair of forceps were rubbed beneath the pony's tongue and then squeezed out into 2 ml. of sterile 0·9 per cent saline. The saline-saliva mixture was boiled for 10 minutes, centrifuged and the supernatant removed and stored at —20°C. The dilution of these samples, subsequently used to inhibit the action of various isoagglutinins, was hard to estimate but was probably in the region of 1:20.

Serological Tests

The direct agglutination test. The first attempts at determining the extent of cross reactions between samples of horse cells and sera were carried out by the methods commonly used for grouping human blood samples. One drop of the 2 per cent. cell suspension was placed in a small tube (an Rh Tube 5 × 50 mm.) and to it was added 1 drop of undiluted, heated serum. The contents were mixed by tapping the tube and allowed to settle for an hour at room temperature; the results were read microscopically. The frequent presence of 'nonspecific autoagglutination' and rouleaux formation made the reading very difficult and the test was later modified to deal with this (described below; "Results" paragraph 2). In later tests the tubes were centrifuged for 30 seconds before reading; by this means a firmer agglutination was obtained.

The direct agglutination test in albumin medium. This test method, as described by Diamond and Denton (1945) was also used to investigate the titres of the naturally occuring isoagglutinins.

The direct agglutination test using trypsin-treated red cells. The red cell suspensions were prepared as described by Morton and Pickles (1951) and used in the direct agglutination test as described above.

The indirect anti-globulin sensitisation test. The anti-horse globulin serum was produced in rabbits (Coombs, Mourant and Race; 1945), absorbed free of horse cell agglutinins and used, after test titrations, at the optimum dilution. Samples of bovine anti-horse globulin serum were later absorbed and tested in parallel with the rabbit anti-horse globulin serum and found to be satisfactory at the dilution of 1:200. This test was carried out in Rh tubes using one drop of 2 per cent. cell suspension and 1 drop of serum. After incubation at room temperature for at least 30 minutes, the cells were deposited by centrifugation; washed 3 times in saline and finally resuspended in 1 drop of diluted anti-globulin serum. The tubes were re-incubated at 37°C. for an hour and the results read macroscopically.

The haemolytic test. The test was performed in Rh tubes. One drop of a 1 per cent cell suspension was mixed with one drop of heated serum and left at bench temperature for 30 minutes. The tubes were then shaken, filled up with 0·9 per cent. saline and centrifuged to deposit the cells. The supernatant was removed and one drop of guinea pig complement, absorbed free of horse agglutinins at 4°C., diluted 1:5, 1:10, or 1:20, was added. The tubes were again shaken and incubated at 37°C. and read after one hour, during which time they were shaken three or four times.

Absorptions. As a general rule, the absorptions were carried out with cells that had been washed three times with 0·9 per cent saline and packed by rapid centrifugation for 10 minutes. Aliquots of undiluted

serum were each absorbed twice with an equal volume of packed red cells for 15 minutes at room temperature. After each absorption, the cells were repacked by centrifugation for 10 minutes. The absorbed sera were either tested immediately or stored at −20°C. Any deviations from this procedure will be noted in the text.

RESULTS

Direct Agglutination Tests

The first step in these investigations was to complete a table of the interactions, recorded by the direct agglutination technique, between the cells and sera of 15 horses, randomly chosen. Table 1 shows the results obtained. On re-arrangement (Table 2), the pattern obtained could be explained by the presence of two separate antibodies occurring singly or together in a particular serum sample and so defining two antigens. These antigens were provisionally and arbitrarily called I and II, recognised by naturally occurring isoagglutinins, anti-I and anti-II. There appears to be a reciprocal relationship between the antigen and its antibody, in that a horse lacking, say, antigen I, invariably possesses anti-I. Four types of blood groups are therefore recognised, and one pony was chosen to represent each of the 4 types.

Cells	*Serum*	*Pony*
Antigen I	anti-II	Henry
Antigen II	anti-I	Arthur
Antigens I plus II	neither antibody	Sophie
Neither antigen	anti-I plus anti-II	Hester

The purity of the antisera containing antibodies I or II, and the separate identity of both these antibodies in single samples of serum was established by a series of absorptions. Aliquots of each of the 3 types of antisera, i.e. anti-I, anti-II, anti-I plus anti-II, were each absorbed with samples of antigen I, antigen II and antigen I plus II cells and also negative cells as a dilution control. The absorbed aliquots were then tested back on the cell panel, using the direct agglutination test (Table 3). It was concluded from these results that the four types of blood groups existed as postulated. In all later tests, serum samples from Henry and Arthur provided the test samples for anti-I and anti-II.

Titre and Reactivity of Anti-I and Anti-II Normal iso-Antibodies

The titre of both the naturally occurring isoagglutinins against both types of cells i.e. I or II and I plus II, was investigated by the following methods:—(a) direct agglutination, (b) direct agglutination in albumin medium, (c) direct agglutination using trypsin treated red cells, (d) indirect anti-globulin sensitisation test, (e) haemolytic test. The results of the haemolytic test were negative. On the other hand, a titre of not more than 1:8 for anti-II and not more than 1:4 for anti-I was obtained by all other methods. The titres of many samples of serum were as low as 1:2. Herman (1936)

TABLE 1

REACTIONS BETWEEN CELLS AND SERA OF 15 HORSES TESTED BY DIRECT AGGLUTINATION TECHNIQUE

	Cells														
Sera	*L.*	*Ba.*	*Be.*	*E.*	*Ed.*	*Pen.*	*S.*	*M.*	*Hes.*	*A.*	*S.L.*	*Hen.*	*San.*	*Peg.*	*Brid.*
Lionelle	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Barbara	—	—	—	+	—	+	+	+	—	—	—	+	+	+	—
Bess	+	+	—	+	+	—	+	+	—	+	—	+	—		
Emption	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Edinburgh	+	—	—	+	—	+	+	+	—	—	—	+	+	—	—
Penny	+	+	—	+	+	—	+	+	—	+	—	—	—	+	+
Sophie	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Morgan	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Hester	+	+	—	+	+	+	+	+	—	+		+	+	+	
Arthur	+	—	—	+	—	+	+	+	—	—	—	+	+	+	+
Steady Light	+	+	—	+	+	+	+	+	—	+	—			+	+
Henry	+	+	—	+	+	—	+	+	—	+	—	—	—	+	+
Sandra	+	+	—	+	+	—	+	+	—	+	—	—	—	+	—
Peggy	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bridget	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

\+ = agglutination.
— = no agglutination.

and Balakrishnan and Veravdekar (1949, 1950) have recorded titres of between 1:8 and 1:12 for samples of horse serum reacting with horse cells. There was no difference between the titres of any antiserum obtained against both types of cells mentioned above; that is to say that no dosage effect of either antigen could be demonstrated.

The method that was found most satisfactory for the grouping of a sample of horse blood was as follows. One drop of a 2 per cent. suspension of red cells is added to one drop of undiluted heated serum in an 'Rh' tube. The contents of the tube are mixed by tapping and the cells left to settle for an hour at room temperature. The tubes are centrifuged at 2000 revolutions per minute for 30 seconds before the results are read microscopically. The contents of the tube are removed very gently with a Pasteur pipette and placed on a grease-free slide with a drop of saline. The mixture is spread firmly by moving the pipette horizontally across the slide, so that all rouleaux formations are broken up. A true agglutination will not be dispersed by this method, though the larger clumps of cells may be broken up into smaller ones. It is essential to test both the cells and the sera with known reagents and to include, as controls, cells whose type are known. In testing eight samples of anti-I and seven samples of anti-II, the titre of the anti-I has always been lower than that of anti-II and the interaction of anti-I and its antigen has always been the weaker reaction.

The method outlined above has been used to type the 52 horses available at present, with the following results:—

Antigens carried on the cell	*No. of horses*
Antigen I alone	7
Antigen II alone	8
Antigens I plus II	21
Neither antigen	16

The number of horses tested is as yet too small to allow for further comments on the distribution of the groups. In 6 cases, the expected agglutinin, anti-II, could not be demonstrated in the sera of samples whose cells carried either antigen I alone or neither antigen.

The Relationship of Equine Antigens I and II to the Human Antigens A and B

Bearing in mind that the majority of the workers mentioned in the introduction of this study classified horse cells as A, B or O without clearly indicating the relationship to the human A, B and O antigens and that both Herman and Guermaine demonstrated that horse serum reacted specifically with human A and B cells, it seemed necessary to investigate further the possible relationship of the named human antigens and the equine antigens here defined by naturally occurring isoagglutinins. It is well known that both A and B substance may be obtained from the gastric mucosa of

TABLE 2

A REARRANGEMENT OF TABLE I TO SHOW THE ANTIGENIC PATTERN

	Antigens															
		I plus II							*I*			*II*			*Neither*		
Antibodies		*L.*	*E.*	*S.*	*M.*	*Peg.*	*Bri.*	*Pen.*	*Hen.*	*San.*	*Ba.*	*A.*	*Ed.*	*Be.*	*Hes.*	*S.L.*
	L.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	E.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	S.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	M.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Peg.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bri.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Anti I	Ba.	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Anti I	A.	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Anti I	Ed.	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Anti II	Pen.	+	+	+	+	+	+	—	—	—	+	+	+	—	—	—
Anti II	Hen.	+	+	+	+	+	+	—	—	—	+	+	+	—	—	—
Anti II	San.	+	+	+	+	+	+	—	—	—	+	+	+	—	—	—
Anti I plus II	Be.	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
Anti I plus II	Hes.	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
Anti I plus II	S.L.	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—

horses and from horse saliva (Friedenreich and Thyssen, 1937; Baer, Kabat and Knaub, 1950), but apparently neither of these substances has been used in an attempt to inhibit horse isoagglutinins and so establish the specificity of the reaction between a particular horse isoagglutinin and a selection of horse cells. The following tests were designed to clarify the relationship between antigens I and II and human antigens A and B, and to verify that horse sera contain the agglutinins corresponding to human anti-A and anti-B:—

(a) After absorption with human A, B and O cells, the titre of the horse isoagglutinins anti-I and anti-II did not differ significantly from the pre-absorption titre against horse cells. It was concluded that human A, B and O cells will not absorb horse agglutinins anti-I and anti-II.

(b) After absorption with horse cells of types I plus II, I, II and negative, no fall was demonstrated in the titre of the post-absorption samples of human anti-A and anti-B sera. It was therefore concluded that horse cells carrying antigens I and/or II will not specifically absorb human grouping antisera.

(c) Twenty-two samples of horse saliva were tested for their ability to inhibit human anti-A and anti-B sera, using the direct agglutination technique. Twelve samples specifically inhibited anti-A, six anti-B and four both anti-A and anti-B. The test was controlled by human A and B secretor and non-secretor salivas. Later tests, again using the direct agglutination technique showed that horse anti-I and anti-II sera were not inhibited by human or horse secretor salivas of any type. The results therefore support the view suggested by the previous experiments that antigen-I and II, defined by naturally occurring horse isoagglutinins anti-I and anti-II are not related to the human A and B antigens.

(d) An attempt to type horse cells for the presence of the A and B antigens was not successful, as there was no reaction between samples of human grouping sera used at a dilution of 1:5 to avoid intra-species reactions, and 10 samples of horse cells chosen at random.

(e) The occurrence in horse serum of agglutinins corresponding to anti-A and anti-B, apart from naturally occurring anti-I and anti-II, was investigated as follows. Twelve samples of horse serum chosen at random, were each absorbed with human group O cells and then tested against a panel of human A, B and O cells. Before absorption, strong species agglutinins accounted for the reaction between human cells and horse serum. After absorption, the reaction of all the absorbed sera with the absorbing cells was negative, but four samples of serum reacted with A cells alone, one with B cells alone and three with both A and B cells. The remaining four samples did not react with any of the cell panel. In order to verify the specificity of these reactions, an attempt was made to inhibit the reactions of each absorbed serum sample with human A and B

TABLE 3

REACTIONS BEFORE AND AFTER ABSORBTION OF SERA CONTAINING NATURALLY OCCURRING ANTI-I, ANTI-II AND ANTI-I PLUS II

Serum samples	*Test cells*							
	Hen.	*Arth.*	*Soph.*	*Hes.*	*Pen.*	*Ba.*	*Br.*	*Bebe*
	I	*II*	*I* *II*	—	*I*	*II*	*I* *II*	—
Anti-I (Arthur) unabs.	+	—	+	—	+	—	+	—
,, abs with Henry	—	—	—	—	—	—	—	—
,, ,, ,, Arthur	+	—	+	—	+	—	+	—
,, ,, ,, Sophie	—	—	—	—	—	—	—	—
,, ,, ,, Hester	+	—	+	—	+	—	+	—
Anti-II (Henry) unabs.	—	+	+	—	—	+	+	—
,, abs. with Henry	—	+	+	—	—	+	+	—
,, ,, ,, Arthur	—	—	—	—	—	—	—	—
,, ,, ,, Sophie	—	—	—	—	—	—	—	—
,, ,, ,, Hester	—	+	+	—	—	+	+	—
Anti-I plus II (Hester) unabs.	+	+	+	—	+	+	+	—
,, abs. with Henry	—	+	+	—	—	+	+	—
,, ,, ,, Arthur	+	—	+	—	+	—	+	—
,, ,, ,, Sophie	—	—	—	—	—	—	—	—
,, ,, ,, Hester	+	+	+	—	+	+	+	—

secretor salivas. Those sera which reacted with A cells alone were inhibited by A secretor saliva only and similarly the serum which reacted with B cells was inhibited by B saliva, i.e. each serum was specifically inhibited. It therefore appears that horse serum may contain agglutinins corresponding to human anti-A and anti-B, but existing independently of the isoagglutinins anti-I and anti-II.

It is of interest to report the 'saliva' type of these twelve particular horses, in order to demonstrate that there is apparently no constant relationship either between the possession of blood group substances in the saliva and agglutinins to human A and B cells in the serum, or the specificity of both these entities when they are together present in one animal.

	Horse	*Agglutinins in serum*		*Saliva type*
1.	Nancy	Anti-A	Anti-I plus -II	AB
2.	Sarah	Anti-A	Negative	B
3.	Percy	Anti-A	Anti-I	A
4.	Arthur	Anti-A	Anti-I	AB
5.	Bebe	Anti-B	Anti-I plus -II	AB
6.	Betsy	Anti-A and -B	Anti-I plus -II	A
7.	Keith	Anti-A and -B	Anti-I	A
8.	Henry	Anti-A and -B	Anti-II	A
9.	Brunette	—	Anti-II	A
10.	Margaret	—	Anti-I plus -II	A
11.	Princess	—	Anti-I plus -II	A
12.	Gert	—	Anti-I	A

The presence of, for example, A substance in the saliva and anti-A agglutinin in the serum of the same animal has been demonstrated in cattle as well as horses (Hartmann, 1941; Wiener, Candela and Goss, 1942). These findings may be explained by the theory of multiple specificities described by Friedenreich (1935) which also explains the incomplete interaction between the 'A' antigen found in pigs and man and the 'B' antigen in rabbits and man.

CONCLUSIONS

Previous work on the blood groups of the horse has been complicated by the initial adoption of the ABO nomenclature of man and the subsequent confusion over the relationship between the groups found in the horse and the human ABO system.

The present investigation confirms that 4 main blood groups may be recognised in the horse with the aid of two natural isoagglutinins which have been called anti-I and anti-II. Group one contains antigen I on the red cells and anti-II in the serum; group two, antigen II on the red cells and anti-I in the serum; group three, antigen I and II on the red cells but neither anti-I nor anti-II in the serum, while group four has neither antigen I nor II on the red cells and both anti-I and anti-II in the serum.

Antigens I and II and their corresponding iso-antibodies are not related in specificity to the human A and B blood group antigens. As is well known, horse saliva may contain substances specifically neutralizing human anti-A or anti-B, but these substances have no inhibitory action on the iso-agglutinins anti-I or anti-II.

Independent of antigens I and II, no antigens reacting specifically with human anti-A or anti-B could be shown on the red cells of 10 horses examined. On the other hand, independent of the iso-agglutininins anti-I and anti-II individual horse sera may contain antibodies reacting specifically with either or both of the human A or B red cell antigens.

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STUDIES ON THE TOLERANCE OF N'DAMA CATTLE TO TRYPANOSOMIASIS*

By

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INTRODUCTION

The tolerance of N'dama cattle to trypanosomiasis, when exposed to natural infection in the field, has been previously reported (Chandler 1952). The available evidence suggested that this tolerance was probably an inherent quality of the breed and not merely a partial immunity acquired as the result of previous infection. The present studies concern the results of challenging with homologous and heterologous strains of *T. vivax* and *T. congolense* those N'dama cattle previously exposed to infection and further animals of the same breed which had not previously been exposed.

MATERIALS AND METHODS

Cattle. The animals were divided as follows. Group I: five N'dama cattle (Nos. 128, 131, 138, 157 and 156) previously exposed to the risk of infection in tsetse areas (Chandler, 1952). Group II: two N'dama cattle (Nos. 198 and 200) and one Gold Coast bull (No. 317) which had been born and bred in fly-proof quarters and not previously exposed to infection. Group III: three calves not previously exposed to infection. Calves Nos. i and ii were believed to be N'dama X Zebu cross-breds, Calf No. iii was believed to be N'dama pure-bred.

Challenges. Challenges with *T. vivax* were made using tsetse fly (G. palpalis) that had been infected under laboratory conditions. One challenge was made using a strain which was geographically homologous to that strain against which the cattle in Group I had previously been exposed under field conditions (Lumu strain): the other challenge made with *T. vivax* utilised a strain which had been obtained from a different locality and which was likely to be heterologous (Toro strain). Challenges with *T. congolense* were made by subcutaneous inoculation of blood from infected laboratory mice or rats. A strain that was geographically homologous (strain B) and a strain likely to be heterologous (strain A) constituted these challenges. Both strains had been recently isolated.

In the case of each experimental challenge, one Zebu cow was challenged along with the N'dama cattle, as an indicator of the effectivity of the challenge, in the following order: first challenge with homologous strain of *T. vivax*; second challenge with heterologous strain of *T. vivax*; third challenge with homologous strain of *T. congolense*; fourth challenge with heterologous strain of *T. congolense.*

It will be noted that the challenges using *T. vivax* were made using infected tsetse fly, while the *T. congolense* challenges were made using infected mouse blood which does not so closely simulate natural challenge.

Serological tests. (T. Congolense). Tests were carried out at various

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intervals on the sera of all cattle under experiment. The lysis-protection test was developed from those described by Schilling and Neumann (1932) and by Fiennes (1950). Blood from a heavily infected mouse was suspended in citrate-Ringer-glucose and added to the test sera. The mixture was then incubated at 37°C for 1 hour; each serological tube was provided with a glass cap and inverted at intervals to maintain the suspension. After incubation, "wet preparations" were made of the tube contents to observe any lysis of the trypanosomes due to antibody. The contents of each tube were then inoculated into mice intraperitoneally. Subsequent infection of the mice with *T. congolense* indicated that protective qualities were absent from the test sera. Controls, where saline replaced the bovine serum, were used throughout.

The lysis test consisted of the first stage of the above test and was used in cases of shortage of mice.

The lysis test with prolonged incubation consisted of the lysis test where the period of incubation at 37°C was maintained for from 4 to 8 hours. This method yielded results which were unobtainable by the standard lysis test.

Serological tests (T. Vivax). The lysis protection test using *T. vivax* established in mice by Unsworth (1953). The lysis test using *T. vivax* established in rats by Desowitz and Watson (1953). Both these strains of *T. vivax* were obtained from areas distant from the area of the cattle's field exposure and were therefore likely to be heterologous.

RESULTS

Before the Challenges

The five N'dama cattle of group I were kept at Vom, a tsetse-free area, from June 1951 to October 1952 being grazed during the day and housed at night. During this 16 months period, which covered both wet and dry season extremes of climate at an altitude of 4,000 feet, the cattle not only maintained their health, but improved greatly in condition, the bulls particularly growing and fattening well; all three cows produced strong healthy calves. Blood examinations were made daily, except Sundays, over this period and, with the exception of 131 which showed scanty trypanosomes for a short period after its arrival at Vom, no trypanosomes were detected. Citrated blood was taken from each N'dama cow and the whole inoculated intravenously into a normal Zebu cow; daily blood examinations for three months failed to detect any trypanosomes in this animal. The N'dama cattle were, therefore, apparently free from infection at the commencement of the present experiments. The two other N'dama cattle and the Gold Coast bull, obtained in June 1952 from Ilorin Stock Farm where they had been born and bred in tsetse-free quarters, similarly did very well at Vom.

Serological tests were conducted on all the cattle on various occasions. *T. congolense*, strains A and B, were used in lysis-protection tests; *T. congolense*, strain B, in lysis tests with prolonged incubation; and *T. vivax*, both strains, in lysis tests. Antibody was detected in

the serum of N'dama 131 against *T. congolense* strain B, using the prolonged incubation test, and against both strains of *T. vivax*. Antibody was not detected in the sera of any of the other cattle.

After the First Challenge (*Homologous Strain of* T. vivax.)

Blood examinations were made daily on all the cattle for two months after this challenge but, with the exception of the Zebu control which showed a low grade and transient infection, no trypanosomes were detected. As dissections showed that none of the surviving flies were infected, the control Zebu must have been infected by one or more of the flies which died before the dissections were made. It may be said, therefore, that the challenge was of low intensity. That the N'dama and other cattle of groups I, II and III showed no infection indicates either that the challenge was insufficient or that these cattle had a degree of resistance.

After the Second Challenge (*Heterologous Strain of* T. vivax)

The infections that occurred in the cattle are recorded in Table 1 from which it will be seen that the N'dama cattle of Group I showed far less intense and less prolonged infections that those of the other groups or the control Zebu. In Group II, 198 responded similarly to the Group I cattle, but in 200 trypanosomes were detectable over a longer period; the Gold Coast bull showed fairly regular but scanty infections on blood examination. All three calves of group III showed similar infections. The control Zebu suffered a more intense infection than any of the other cattle. None of the cattle showed clinical signs of the disease except the control Zebu which became emaciated 3 months after the challenge.

It may be summarised that the five N'damas previously exposed in the field rapidly eliminated the parasites from the peripheral circulation and one N'dama never showed a blood infection; this suggests that their previous exposure to infection enhanced their resistance to the present challenge. There were indications that the N'dama cattle of Group II, which had not been previously exposed, were more liable to be affected by a *T. vivax* challenge. The course of infection in the Zebu control indicated that the other cattle breeds had a measure of tolerance when compared with the Zebu.

Serological tests of the lysis type were conducted. All the cattle of groups I, II and III possessed antibody to the two strains of *T. vivax* used, one homologous and one heterologous to the challenge strain. There was no cross-immunity with *T. congolense*, suggesting that the immune response was species specific. The Zebu control possessed a lower level of antibody than that of the other cattle.

After the Third Challenge (*Homologous Strain of* T. congolense)

The results of blood examinations made daily on all the cattle are recorded in Table 2, where it will be seen that all the cattle developed infections of medium intensity until 50 days after

Table 1. Results following challenge with T Vivax (Toro Strain)

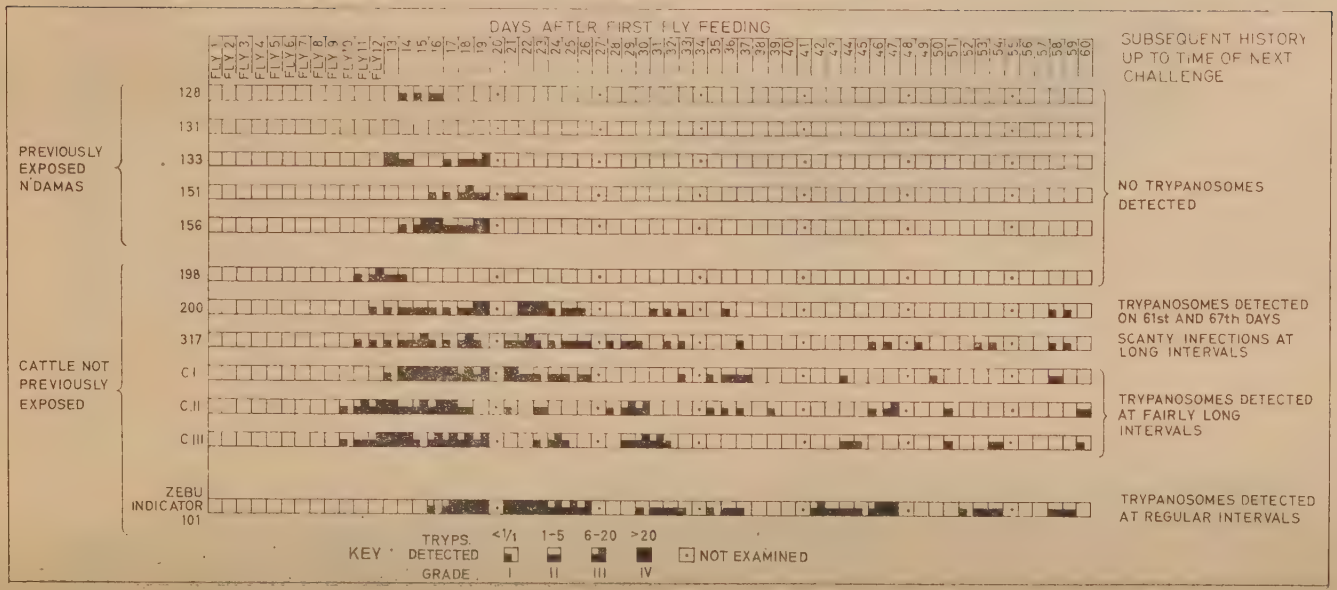
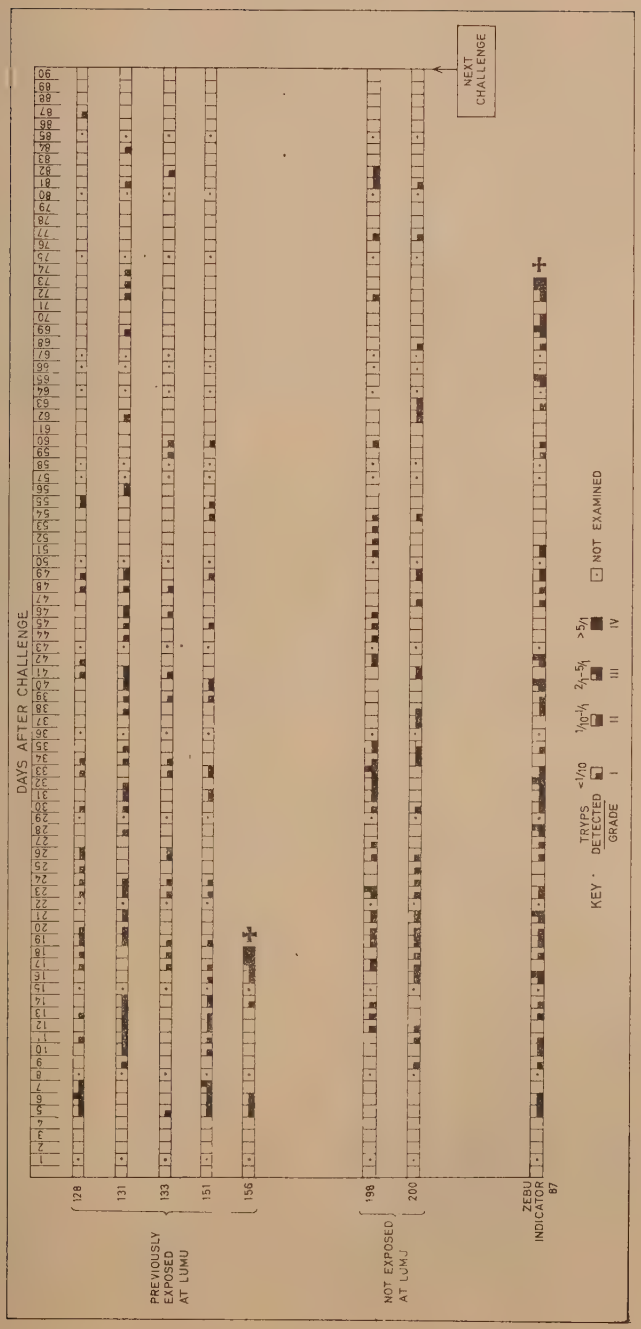


Table II. Results following T Congolense (Strain B) Challenge



challenge, with the exception of N'dama cow, 156, which died of causes other than trypanosomiasis. The numbers of trypanosomes present on examination subsequently became very low, except in the case of the Zebu control. There was no noticeable difference in the course of infection in the Group I or Group II cattle. The Zebu control died of trypanosomiasis, whereas the other cattle showed no clinical signs of disease.

Serological tests of the lysis-protection type showed that all cattle, including the Zebu control, developed antibody to both homologous and heterologous strains of *T. congolense*; a limited test indicated that the lytic antibody was present to the same degree in both the N'dama cattle and the Zebu control. Tests made 3 months after the challenge showed that the antibody titre had not declined. Tests using *T. vivax* showed that all the cattle previously challenged with *T. vivax* had retained their antibodies to this species.

After the Fourth Challenge (*Heterologous Strain of* T. congolense)

The results of blood examinations are recorded in Table 3, where it will be seen that infections occurring in the N'dama cattle of Groups I and II were of a very low intensity. Infections in the Gold Coast bull were of somewhat higher intensity, but still less than those in the calves and Zebu control. Fifty days after the challenge all the cattle were apparently in good clinical health except the Zebu control which had lost weight considerably.

Serological tests demonstrated the presence of antibody to the *T. congolense* strain used in this challenge in the sera of all the cattle used.

CONCLUSIONS

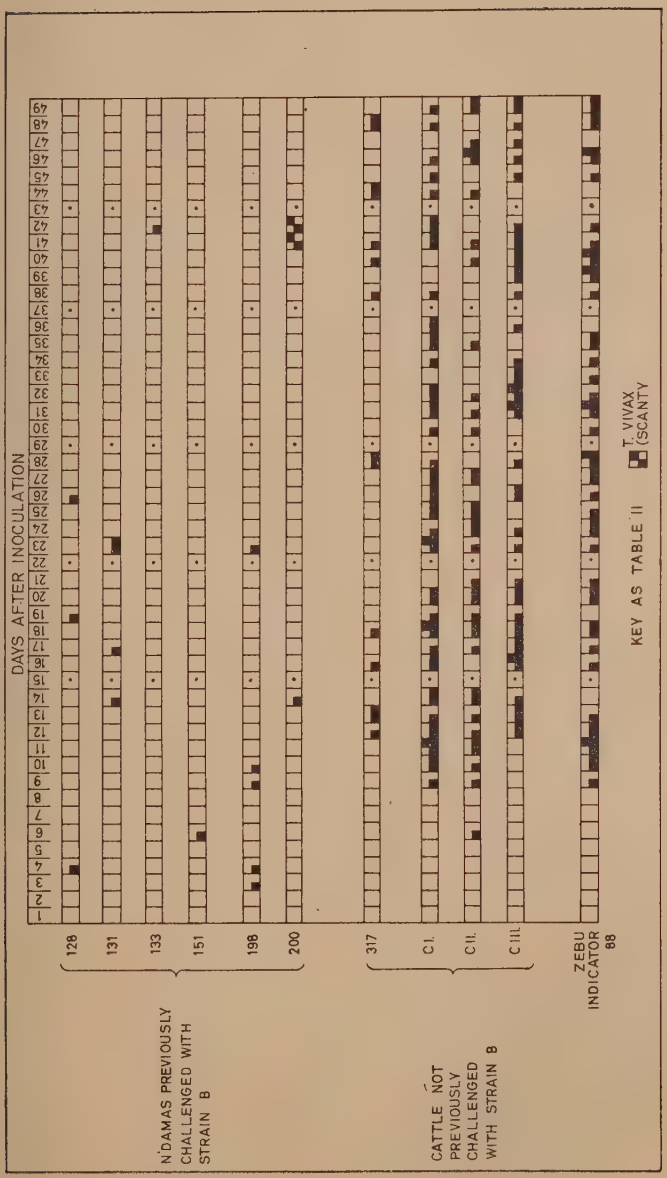
N'dama cattle, which had previously been exposed to trypanosomiasis, not only maintained their health but improved in condition when exposed to the stress of seasonal extremes of climate in an area 4,000 feet above sea-level. The cattle apparently eliminated trypanosomes from their peripheral circulation. Serological studies revealed no evidence of specific antibodies in the sera of four of the five cattle.

Following fly-challenge with *T. vivax*, the previously exposed N'dama cattle showed less intense and less prolonged infections than cattle which had not been previously exposed, particularly a Zebu control. Serological tests showed that the cattle developed antibody to two strains of *T. vivax* but not to a strain of *T. congolense*.

Challenges with *T. congolense*, using infected blood, resulted in infections which subsequently declined in the N'dama cattle but caused clinical disease in the Zebu controls. Antibodies were produced against *T. congolense* and these were not strain-specific.

The evidence suggests that the N'dama breed of cattle possesses a degree of tolerance to the pathogenic effects of infections with a

Table III. Results following T Congolense (Strain A) Challenge


DAYS AFTER INOCULATION
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49
N'DAMAS PREVIOUSLY CHALLENGED WITH STRAIN B
128
131
133
151
198
200
CATTLE NOT PREVIOUSLY CHALLENGED WITH STRAIN B
317
C I.
C II.
C III.
ZEBU INDICATOR 88
KEY AS TABLE II
T. VIVAX (SCANTY)

number of strains of different geographical origin, of both *T. vivax* and *T. congolense*. The serological studies suggest that specific antibodies are not elaborated except as a response to infection and that they are species-specific.

The present studies, together with those previously reported, indicate that tolerance to trypanosome infection is an inherent quality of N'dama cattle; there are in addition, indications that exposure to infection enhances this tolerance. The sum of evidence is sufficiently convincing to justify the limited use of N'dama cattle in tsetse areas in West Africa, but only practical experience will show whether this tolerance will prove to be effective under various conditions in the field.

It is suggested that, should chemoprophylactic or chemotherapeutic measures be required under certain circumstances, these are more likely to be effective in N'dama cattle than in Zebu cattle, since their effect would be reinforced by the degree of tolerance possessed by N'dama cattle.

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STUDIES ON CATTLE PROTECTED BY ANTRYCIDE AND EXPOSED TO REGULAR CHALLENGES WITH TRYPANOSOMES*

By

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INTRODUCTION

Studies by Unsworth and Chandler (1952) indicated that Zebu cattle, protected by Prophylactic Antrycide**, and exposed to a relatively light trypanosome challenge in the dry season, withstood a heavier challenge in the rainy season better than similarly protected cattle which were initially exposed during the rains. This observation suggested that the protective action of the drug might be reinforced by immunity, acquired as the result of repeated antigenic stimulation of the bovine host by bites of infective tsetse. The present studies, carried out at Vom which is a tsetse-free area, were designed to investigate this hypothetical immunity, under conditions in which the degree of exposure to infection could be controlled.

MATERIALS AND METHODS

Cattle. Zebu cattle (18) were used and divided into two groups, I and II, each of nine cattle.

Drug administration. All 18 cattle were inoculated with Antrycide Pro-Salt** in the recommended dosage (15 ml. for a 300 kg. beast), given subcutaneously in the left side of the neck. Whenever subsequent serum samples were taken or trypanosomes inoculated, the right side of the cattle was used, in order to avoid accidental contact with Antrycide in the depot.

Blood examinations. Prior to commencement of the experiment, blood examinations were made daily on all cattle for one month and subsequently at frequent intervals for a further three months; no trypanosomes were detected. After commencement of the experiment, blood examinations were made, on two consecutive days a week, on all cattle which had been challenged with trypanosomes in any way, and daily for six weeks after major challenges.

Trypanosome strains. The main trypanosome strain used to challenge the cattle, and in the conduct of serological tests, was *T. congolense* strain B; a further strain, *T. congolense* strain A, which was heterologous to strain B, was also used in the conduct of serological tests. Both strains had been recently isolated from different localities in Nigeria and were maintained in mice. A branch strain of *T. congolense* strain A, possessing a high degree of resistance to Antrycide, was used as a final assault challenge towards the end of the experiment.

*Part of a Thesis for degree of Ph.D., University of London.

**Trade Mark of Imperial Chemical Industries.

Routine regular challenges of group I cattle. At each regular challenge each bovine was inoculated subcutaneously with a suspension containing approximately 1 to 5 million trypanosomes of *T. congolense* strain B; a trial experiment had shown that a bovine was infected using this intensity of challenge.

Assault challenges. Three assault challenges were made using *T. congolense* strain B in various cattle of the two groups; at each challenge each cow was inoculated with from 130 to 180 million trypanosomes and the effectivity of each challenge controlled using additional cattle to those of the main experiment.

Serological tests. Tests, the techniques of which have been described by Chandler (1958), were conducted throughout the experiment.

Plan of experiment. The group I cattle were used to determine, by comparison with those of group II, whether an immune response developed subsequent to the regular repeated challenges, these challenges simulating the effects of regular exposure to tsetse. The group I cattle were challenged, in the first instance one week after Antrycide administration and thence once weekly for twelve weeks. The group I cattle were then divided into three groups; one of these groups was subjected to an assault challenge at 3 months, one at 6 months and one at 10 months after Antrycide administration. The results of these challenges, when compared with similar assault challenges made of the group II cattle, would indicate whether any immunity had been acquired in the group I cattle, as a result of the regular routine challenges which enhanced the protective action of Antrycide. Regular routine challenges were reinstituted on all group I cattle that had been assault challenged one month after the assault challenges. Eleven months after the original Antrycide administration, an assault challenge was made using the branch strain *T. congolense* strain A which possessed resistance to Antrycide.

RESULTS

Prior to the Assault Challenges

Serological tests showed that group I cattle, as a result of the routine regular challenges, developed immune body to the homologous strain only; this immune body was first detected in two animals four weeks after the commencement of the regular challenges. No immune body was detected in the group II cattle.

Following the First Assault Challenge

Regular blood examinations failed to reveal infection in either group of cattle: normal cattle, which were inoculated as controls of the effectivity of the challenge, all developed infections. It was concluded that the prophylactic action of Antrycide alone was sufficient to protect the experimental cattle. Serological tests showed that the group I cattle possessed immune body, and in those cattle where routine challenges were not reinstituted, the titre of antibody apparently declined.

Following the Second Assault Challenge

No trypanosomes were detected in either group of cattle, whereas

all the normal cattle used as infectivity controls developed infections. It again appeared therefore that the cattle were protected by the Antrycide. Haematological studies consisting of red cell counts, haemoglobin estimations and haematocrit determinations were made on the two groups of cattle; there was no evidence that the group I cattle were suffering from cryptic infection with trypanosomiasis. Serological tests again detected immune body in the group I cattle receiving regular challenges.

Following the Third Assault Challenge

No trypanosomes were detected in either group of experimental cattle, whereas the infectivity control developed infection. The fact that the Antrycide alone was capable of protecting the two groups of cattle for a period of ten months was remarkable and unexpected.

Following the Final Challenge using a Drug-resistant Strain

All the cattle in either group that were challenged with the drug-resistant strain of *T. congolense* showed the presence of trypanosomes in the peripheral circulation within nine days. One month after this challenge all the cattle inoculated showed loss in weight; there was no noticeable difference in the response of the two groups of cattle to infection. It will be noted that the strain used in this challenge was heterologous to that used in the routine regular challenges and in the previous assault challenges.

CONCLUSIONS

Eighteen cattle, all inoculated with the field dose of Antrycide Pro-salt and some regularly challenged with *T. congolense*, were not apparently infected by heavy challenge with *T. congolense* up to ten months after the original drug administration. It was apparent that the drug alone was capable of protecting all the cattle during this long period.

Serological tests were conducted throughout the experiment and antibodies, against the homologous strain only, were detected in the sera of those cattle which were regularly challenged.

All the cattle became infected and lost weight following a final heavy challenge using a strain of *T. congolense* which was both heterologous to that of the previous challenges, and, in addition, resistant to Antrycide.

It was not possible to determine whether as a result of these regular challenges, the regularly challenged cattle possessed any degree of protection against the homologous strain of *T. congolense*; it was however shown that these cattle did not possess any noticeable degree of protection against the heterologous strain.

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THE SKELETAL MUSCLE OF SHEEP AFFECTED WITH SCRAPIE

By

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INTRODUCTION

During the past century, a number of workers have investigated scrapie in an attempt to find characteristic microscopic postmortem changes. These attempts have been singularly unproductive. The predominant change reported by all was emaciation and general loss of condition. May (1868), Cassirer (1898) and McGowan (1914) described the muscles of most animals dying of scrapie as pale and soft, while the surrounding fat was undergoing gelatinous change. Cassirer (1898) and later McFadyean (1918) and Gaiger (1924) attributed the symptoms of weakness to extreme loss of condition and exhaustion. Cassirer dismissed muscle as the primary site of the disease since he could find no evidence of local atrophy. McGowan (1914) on the other hand, attributed the loss of condition and pruritis to a severe infestation of sarcocysts in the muscle. Stockman (1926) refuted this belief and was later supported by Brownlee (1940). Stockman (1913), Bertrand, Carré and Lucam (1937), Greig (1940) and Lucam, Béchade and Saurat (1950) remarked on the absence of significant muscular change and Bertrand *et al.* (1937) further commented on the probability of the central nervous system being the primary site of the disease.

In contrast Bosanquet, Daniel and Parry (1956a) and Parry (1957) reported finding sufficient muscular change in fifty-two cases to support their hypothesis that scrapie was a primary degeneration of skeletal muscle. While they conceded that one of the principal features of the disease was a "slow wasting of body flesh", they stated that in addition to wasted cases, non-wasting forms of the disease were encountered. According to these authors, the symptoms of scrapie can be directly related to muscular lesions and depend on the location, age of development and extent of "myopathic" processes. Lesions were found by naked eye examination and appeared in the form of either intense pallor (focal or diffuse), wasting of muscle, firm lesions or finally as an excessive deposition of fat. These macroscopic lesions showed microscopic changes ranging from destruction of "one fibre per field" to such intense destruction that very few normal fibres could be found in a field. They describe early changes in the muscle fibre as:— rounding, indistinct appearance of striations, increase in sarcolemmal nuclei, fragmentation of the fibres and granular change in the sarcoplasm.

In the opinion of these authors, the irregular distribution of degenerated fibres was quite different from the distribution of degenerated fibres which occurs after interruption of nerve supply. The similarities between the picture presented and that in the human diseases dermatomyositis and progressive muscular dystrophy were remarked upon, but the term "myopathy" was preferred to define a muscle degeneration of unknown cause. The authors presented a series of nine myopathic syndromes which included scrapie.

The purpose of this paper is to present some observations on the skeletal muscle of sheep showing symptoms of natural and experimental scrapie, and to discuss their significance.

MATERIALS AND METHODS

The sheep examined in this survey were from several different areas in Scotland and included Cheviot, Swaledale and Suffolk sheep. The natural cases were selected by shepherds and practising veterinarians and were observed for periods ranging from four days to five months. One animal was destroyed on arrival since it was in extremis.

With the exception of case 48, any animal which failed to show typical symptoms of scrapie while under observation, was discarded, as were animals which on post-mortem examination showed evidence of any disease process which may have complicated the symptoms. Although, case 48 showed only uninterrupted and generalised tremors, it was considered a possible atypical case of scrapie and was included in the present survey as the history of the flock revealed two other probable cases of scrapie and microscopical examination of the medulla showed severe and widespread vacuolation of neurons (Zlotnik, 1957).

The experimental cases, produced by intracerebral or subcutaneous inoculation of brain suspensions of clinically positive sheep, maintained in serial passage, were kept under special observation from the time that first symptoms of pruritis appeared.

The criteria for acceptance of a clinical case were: (a) severe pruritis (with consequent rubbing) and loss of wool in the absence of ectoparasites or other irritants; (b) progressive loss of condition; (c) locomotor difficulty and progressive weakness, in most cases to the point of inability to rise. In addition, many cases showed hyperaesthesia, quivering, severe ataxia and a swaying gait.

This survey includes sixteen natural and fourteen experimental cases of scrapie, and twelve animals which showed no clinical symptoms of the disease. Thirteen of the natural and all fourteen of the experimentally induced cases showed advanced clinical symptoms and most of them were killed when death seemed imminent. Three were killed three to ten weeks after the appearance of symptoms. Evisceration and skinning were carried out within one or two hours and the carcasses left in a cool room or cold-box for four to twenty-four hours to allow the muscle to "set". During the gross examination, each muscle was separated at origin and insertion and carefully inspected for abnormalities. Next, each was examined after slicing transversely or longitudinally into approximately half-inch slices. The samples of muscle selected for histological examination included any hæmorrhagic portions and any areas which contained a streaking. In this manner, each case yielded sixteen to ninety-four samples from different areas, although in some cases more than one sample was taken from a single muscle.

Samples were fixed in formol-saline, imbedded in paraffin and cut at 7 to 10 μ. One section from each sample was stained with haematoxylin and eosin and selected slides were stained by several additional methods, notably phosphotungstic-acid haematoxylin, Giemsa, Kull, van Gieson, von Kossa and Heidenhain's iron haematoxylin.

The control animals used in this survey included animals within the same age range as the natural and experimental cases, and consisted of

apparently normal animals and animals killed or dying of various chronic diseases. Killing, sampling and processing were carried out in a similar manner to that described previously.

RESULTS

Post-mortem Examination

Of the forty-two sheep examined, only one, a natural case of scrapie, showed gross muscular lesions. The carcasses of the remaining forty-one exhibited only variable emaciation ranging from nearly normal to extreme wasting. An estimate of carcass condition is made in column 2, Tables 1, 2 and 3, and it will be seen that no correlation existed between emaciation and histological groupings.

TABLE 1

MUSCLE CHANGES IN NATURAL SCRAPIE

Serial number	*Loss of condition*	*Microscopic examination*			
		Number of muscles examined	*Number of muscles containing changes*		
			Type I	*Type II*	*Type III*
5	Slight	20	8	—	—
7	Slight	20	4	—	—
13	Slight	20	7	—	—
16	Severe	20	—	—	—
21	Moderate	20	8	—	—
22	Slight	20	8	—	—
29	Moderate	20	2	—	—
30	Severe	20	1	—	—
31	Severe	20	2	—	—
32	None	20	5	1	—
35	Severe	20	4	—	—
39	Slight	50	33	—	3
40	None	44	21	—	—
43	Severe	42	4	—	—
44	None	18	5	—	—
*48	Slight	16	9	—	—

Nos. 7, 32, 40, 44—Suffolk
Nos. 5, 13, 16, 29, 31, 43—Cheviot
Nos. 21, 22, 30, 35, 39—Swaledale
No. 48—Cheviot Crossbred.

*No. 48 showed macroscopic lesions and Type IV microscopic changes in 10 muscles.

TABLE 2

MUSCLE CHANGES IN EXPERIMENTAL SCRAPIE

Serial number	*Loss of condition*	*Microscopic examination*			
		Number of muscles examined	*Number of muscles containing changes*		
			Type I	*Type II*	*Type III*
6	Severe	20	1	—	—
8	Moderate	20	18	—	—
9	Slight	20	7	—	—
11	Moderate	22	12	1	—
20	Moderate	20	10	2	—
23	None	20	5	—	—
27	Moderate	20	8	—	—
28	Slight	20	3	—	1
33	Moderate	20	7	—	—
34	Moderate	20	5	—	—
45	Moderate	20	2	—	—
46	Slight	16	3	—	—
47	Slight	16	4	—	—
53	Slight	16	5	—	—

All animals are of the Cheviot breed.

No abnormality whatsoever could be detected in the muscles from four scrapie and six non-scrapie animals. Ten scrapie and three non-scrapie controls showed a slight reduction in volume of skeletal muscle and an apparent reduction in fat cover. Nine scrapie and two control animals showed a marked reduction in fat cover and a moderate reduction in muscle volume. Seven scrapie and one control sheep showed a complete absence of body fat and a marked reduction in muscle volume. The muscles were soft and pale and surrounded by oedematous, gelatinous connective tissue. With regard to wasting, no apparent difference existed between natural and experimentally induced cases of scrapie, and any breed difference was attributed to basic anatomical differences in fat reserves. A feature in four scrapie animals with almost normal carcasses was the presence under the skin of the back of single or multiple necrotic or sero-sanguineous pockets in the subcutaneous fat layer. These lesions varied in extent from small foci 1 cm. in diameter to a large multilocular cavity extending from the sacral

to the cervical region. These were considered to be due to traumatic fat necrosis. A characteristic of the very emaciated carcasses was the absence or incompleteness of rigor mortis, even after several hours in a cool room.

TABLE 3

MUSCLE CHANGES IN NON-SCRAPIE ANIMALS

Serial number	*Loss of condition*	*Microscopic examination*			
		Number of muscles examined	*Number of muscles containing changes*		
			Type I	*Type II*	*Type III*
24	Moderate	7	2	—	—
25	Moderate	4	2	—	—
26	Slight	4	2	—	—
36	Slight	20	8	—	—
37	None	6	1	—	—
38	Slight	20	14	—	—
41	None	6	2	—	—
49	None	24	23	—	—
50	Severe	14	4	—	—
51	None	24	8	—	—
55	None	20	15	—	—
56	None	20	17	—	—

All animals are of the Cheviot breed.

The animal showing gross muscular lesions was in good condition and had a moderate fat cover. Several muscles contained numerous, minute creamy-white foci which, in the more extensively involved muscles, were so dense that the entire muscle had a creamy colour. In some areas the lesions involved only the surface fibres, while in others the entire muscle was involved. In general, these muscles did not appear enlarged, but were somewhat firmer than normal. Muscles showing lesions in bilateral symmetry were:— extensor carpi radialis, supraspinatus and gastrocnemius. Those with a unilateral distribution were:— sub-scapularis, latissimus dorsi, semi-tendinosus and sartorius. The other skeletal muscles did not appear to be involved and although several were sampled, none contained further lesions.

Histology

Since a detailed description of relatively small changes tends to

give them a false importance, a proper perspective might be established by emphasising the minor nature of most changes found in this survey. With the exception of Type IV to be described, none were sufficiently extensive to produce a grossly visible lesion. Again, with the exception of Type IV and the very minor changes of Type I, they were confined to a very few muscles in a small percentage of scrapie cases.

On the basis of differences in distribution within a sample and differences in morphology, the microscopic changes were classified under four groups as below. The numerical grouping does not necessarily denote a progressive increase in extent or severity of the changes. Variable histological atrophy of muscle fibres visible in many scrapie cases and also in some non-scrapie animals bore no relationship to other changes seen, with possible exception of Type II. Particularly in the more emaciated animals, the general reduction in fibre diameter resulted in a condensation of interstitial tissue.

Type I. Changes of single fibre degeneration or focal increase in muscle nuclei were seen in one or more muscles from all but one case of scrapie and in one or more muscles from all non-scrapie animals. This group includes three somewhat different deviations from normal—hyaline degeneration, replacement of cytoplasm by proliferating muscle nuclei and proliferation of muscle nuclei in the absence of any other change. These three deviations can be considered together because their distributions were similar, none appeared to be more than a very minor alteration and all three were visible in apparently normal control animals. Two of the changes, hyaline degeneration and replacement of cytoplasm by muscle nuclei, were usually confined to two or three fibres, rarely up to ten, in a muscle sample containing from five to twenty thousand fibres. The third variation, proliferation of muscle nuclei, involved more fibres but to a lesser degree. The foci occasionally filled a high power field, though the fibres involved showed no other abnormalities.

Hyaline or granulo-fatty fibres appeared swollen, flat and pale when stained with haematoxylin and eosin. Cross and longitudinal striations were absent or greatly distorted, and muscle nuclei were not visible. In those single, isolated fibres where a nuclear reaction had been stimulated, the nuclei replaced the cytoplasm or merely surrounded normal or degenerating cytoplasm. In these latter fibres, a ring of nuclei, one or two deep, often surrounded a central mass of cytoplasm containing a sarcocyst (Fig. 1). Occasionally, the nuclei were vesicular, but more often they were elongated, small and dark staining (Fig. 2). An occasional macrophage was visible in the centre or at the edge of these fibres.

In longitudinal sections, Type I changes seldom involved more than a short segment of a single fibre, although nuclei of adjacent normal fibres frequently multiplied where damaged and normal

fibres touched. The diameter of fibres which were involved varied greatly: some appeared reduced in size, while others were distended and packed with nuclei. In some cases variations of Type I changes apparently represent stages in a single process, but in view of their sparse, though wide, distribution they can be accorded no real significance.

Type II. Degenerative and atrophic changes were seen in one muscle from an animal with natural scrapie and in three muscles from two experimental scrapie cases, but not in muscles from non-scrapie controls. They consisted essentially of a diffuse increase in muscle nuclei over part or all of the sample and, in addition, ten or more fibres showed evidence of cytoplasmic damage. The cellular appearance was due primarily to enlargement and proliferation of nuclei at the borders of the fibres (Fig. 3). Few fibres were completely ringed by nuclei, but some of those that were showed hyaline or granulo-fatty change and a few in each sample were at least partially replaced by macrophages and proliferating muscle nuclei. More than two damaged fibres were seldom seen in a single primary bundle and many primary bundles contained none.

Although there was generally a focal reduction in fibre diameter and a gradual variation down to about ten microns, most fibres in these areas appeared normal in staining reaction and striation. Occasionally, in the connective tissue surrounding primary bundles, aggregations of lymphocytes, macrophages and eosinophils were visible, particularly adjacent to small vessels. Although some condensation of connective tissue was visible, there seemed to be no absolute increase. The distribution and extent of the lesions in this category suggest that they are more than a simple extension of Type I changes, although the focal increase in muscle nuclei of Type I may represent an early stage of a Type II lesion.

Type III. Fibrotic changes were seen in three muscles from one natural and in one sample from one experimental case of scrapie. They were not seen in non-scrapie controls. The lesions were focal in distribution and contained ten or more damaged fibres. A single focus contained up to fifty or sixty fibres showing some degree of replacement of cytoplasm by large vesicular muscle nuclei. Not more than one focus was visible in a single muscle sample. As in Type II, occasional single fibres showed hyaline degeneration or fragmentation of the cytoplasm, but no macrophages or lymphocytes were visible. A characteristic of Type III lesions was an absolute increase in connective tissue between individual fibres which seemed to extend from the heavier connective tissue bands around vessels and nerves (Fig. 4).

Two samples from the natural case showing Type III lesions also showed a vascular change which was visible in or adjacent to the muscular lesions. A small thrombus lay in one artery, while in the other the intimal layer showed signs of reduplication and partially

filled the lumen. Quite different arterial changes, mostly consisting of calcification or scarring of the medial layer, were seen in four other scrapie animals in addition to the case mentioned above. In these there was no apparent muscular change related to the vascular lesions.

Type IV. Dystrophic lesions were seen only in the animal showing gross lesions (number 48) and consisted of widespread and complete necrosis of fibres in the muscles involved. In more severely affected areas, very few recognisable fibres remained, but in adjacent areas the fibres appeared normal in every respect. Occasionally, fibres directly adjacent to damaged fibres did show evidence of fragmentation, or, less frequently, hyaline degeneration, but as a rule the limits of the lesions were sharply defined. The very cellular appearance of the lesions was due to a periphery of large, vesicular muscle nuclei which surrounded an angular mass of degenerating cytoplasm or, more frequently, an amorphous, finely granular mass (Fig. 5). On staining by von Kossa's method, this granular substance proved to be calcareous (Fig. 6). In other fibres, the proliferating nuclei completely filled the space formerly occupied by cytoplasm and these fibres were somewhat larger than normal ones in the same slide. Rarely, macrophages were visible in or adjacent to fibres which still contained fragments of cytoplasm, and in a few areas collections of lymphocytes were seen near small blood vessels.

In two muscles, in addition to the changes described, a single focus, about one mm. in diameter, showed a marked reduction in the diameter of otherwise normal fibres. Peculiar to these areas was an absolute increase in perimyseal connective tissue. There was little evidence to suggest that an inflammatory reaction was involved, and the distribution of destroyed fibres followed no pattern which suggested a neurogenic origin.

DISCUSSION

Previous to 1956, all investigators were unanimous in agreeing that the only significant change in muscle from scrapie sheep was that of emaciation and consequent loss of muscle volume. It is conceivable that some of these workers may not have examined muscle in any great detail, but it seems unlikely that all would have overlooked the gross lesions reported by Bosanquet *et al.* (1956a) and Parry (1957).

The present survey supports the view that muscle lesions are not a characteristic of scrapie and therefore scrapie cannot be considered a primary muscular disease. The apparent equal distribution of Type I changes in scrapie and control animals makes them obviously unimportant in scrapie. Type II and III changes borrow a false significance from the fact that they were seen only in scrapie animals. Their distribution and severity warrant little

significance even as secondary changes in the disease, and they were seen in far too few cases (five out of thirty) to account for any symptoms seen in the disease. Type IV lesions are undoubtedly significant as secondary or incidental changes, but since they were seen in only one animal with scrapie they can in no way explain symptoms or pathology of scrapie generally.

Any comparative discussion of histological changes of skeletal muscle must be tempered by the observations made by Adams, Denny-Brown and Pearson (1954) and Walton and Adams (1956) that muscle has a somewhat limited range of potential reactions, and consequently that a muscle lesion is seldom diagnostic. Although a consideration of the muscle changes reported in this survey must, at this point, be largely speculative, certain features might be pointed out and discussed.

Type I changes involving such widely scattered, single fibres, can scarcely be considered manifestations of a general disease process. The finding of similar changes in all control animals suggests that they are little more than physiologic variations which could be found in any sheep.

Type II changes were found in muscles lying near the surface and in areas where scrapie sheep are frequently seen to rub, namely sternocephalic, semi-tendinosus, semi-membranosus and longissimus dorsi muscles. Such an anatomic distribution makes it tempting to consider them traumatic in origin. Skin and subcutaneous fat often show evidence of traumatic damage, and it is conceivable that muscle could also be involved. On the other hand, the focal reduction in fibre diameter and the increase in muscle nuclei, without evidence of dystrophic change, might suggest an interruption of nerve impulses at some point in the motor chain. The severe ataxia in many scrapie animals, and the variable number of vacuoles found in the brain and cord, point to some deviation from the normal. It would be surprising if the muscle did not occasionally show the effect of these central nervous disturbances. The inverse relationship between nerve continuity and the number of muscle nuclei observed by Tower (1937) is of interest in connection with Type II changes.

The fibrotic Type III lesions contained no features which suggest either a traumatic or a systemic nutritional origin, and the anatomic distribution is of no help here. The proximity of arterial lesions in two examples suggest a relationship, but the assumption that the vascular changes were primary is unwarranted. In connection with the fibrosis, Adams *et al.* (1954) cite reports that experimental nerve section and venous, but probably not arterial occlusion, can produce a fibrous hyperplasia. Tower has further observed that fibrosis around nerves and vessels in disuse atrophy results in a secondary muscular degeneration. In view of the non-specificity of muscular changes generally, it is difficult and probably

inadvisable to attempt a definite classification of Type II and III changes.

On the other hand, the dystrophic, Type IV lesions seen in case 48, present a number of features which allow at least a general classification. The distribution of acutely damaged fibres and the massive calcification of necrotic fibres is similar to, if not identical with, changes in the metabolic disease attributable to a deficiency of vitamin E. The histologic picture presents no features to suggest that anything more is involved, and even the atrophic, fibroitc variations seen in two muscles have been described by Marston and Pierce (1942) as a feature of muscular dystrophy attributed to vitamin E deficiency. Although some similarities exist, an essential difference between Type IV lesions and the "more severe" lesions reported by Bosanquet *et al.* (1956a) and Parry (1957) is the presence of calcium in the damaged areas. Investigation into the history of this sheep revealed nothing unusual about the diet comprising grass and turnips, and no unusual exercise could be traced. It is interesting to note that although the muscle lesions were moderately extensive, the animal clinically showed no indication of stiffness, ataxia or instability. Bosanquet, Daniel and Parry (1956b) point out that vitamin E controlled dystrophy is usually confined to young lambs and calves, but several authors have reported dystrophy in more mature sheep (Marston and Pierce, 1942; Cotchin, 1947; Marr and Sharman, 1956). These last authors also cite a similar report by Dodds (1954), and unpublished reports by Hartley (1954) and Watt (1954). Hadlow (1955) has also reported similar lesions in a wild Virginian deer in the U.S.A.

CONCLUSIONS

Skeletal muscles from thirty cases of scrapie, fourteen experimentally induced and sixteen natural, have been minutely examined and compared with muscles from twelve non-scrapie control sheep.

Macroscopic lesions were found in one natural scrapie sheep and minor microscopic changes were found in five more, two natural and three experimental.

It is concluded that scrapie cannot be considered a primary disease of the muscle, and that muscle changes play no apparent part in producing clinical symptoms of that disease.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Type I change surrounding a fibre containing a small sarcocyst. A second sarcocyst in the upper part shows no accompanying cellular increase. × 320.

Fig. 2. Type I change showing proliferating muscle nuclei replacing muscle substance. × 320.

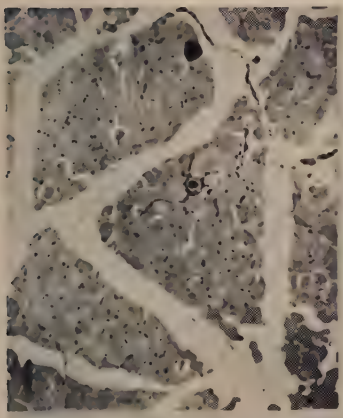
Fig. 3. Type II change showing a general reduction in fibre diameter and a diffuse increase in muscle nuclei. × 320.

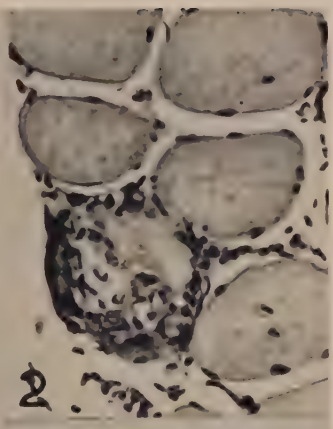
Fig. 4. Type III fibrotic lesion showing massive increase in perimyseal connective tissue. × 130.

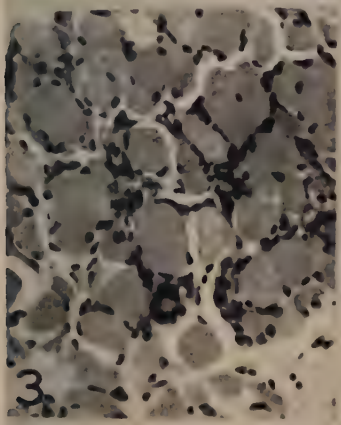
Fig. 5. Type IV lesion demonstrating the severe destruction of muscle fibres and the granular calcification of necrotic fibres. × 320.

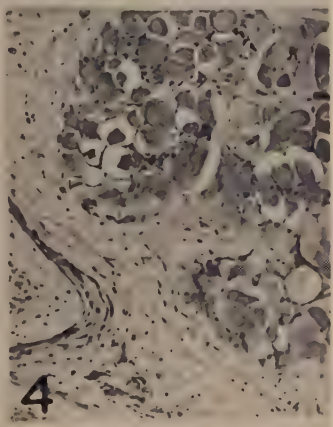
Fig. 6. Type IV change stained to show calcification. × 320.

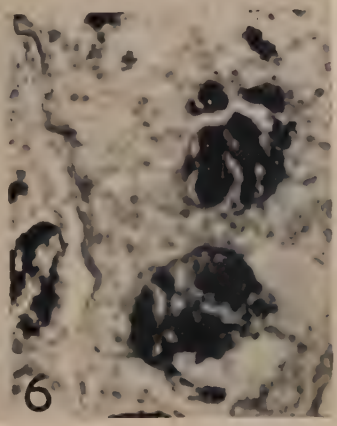
Photomicrographs 1 to 5 have been made from slides stained with haematoxylin and eosin. Number 6 is of a slide stained by von Kossa's and van Gieson's methods. All are from advanced cases of scrapie.











To face page 274

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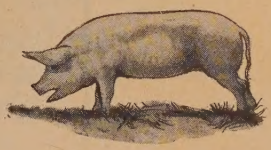

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